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PRINCIPAL INVESTIGATOR: David A. Gewirtz

CONTRACTING ORGANIZATION: Virginia Commonwealth University
Richmond, VA 23284

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David A. Gewirtz

E-Mail:david.gewirtz@vcuhealth.org

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Virginia Commonwealth University; 800 East Leigh St;P.O. Box 980568; Richmond, VA 23298-0568

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14. ABSTRACT **Despite advances in cancer therapeutics over recent decades, developing effective strategies for the treatment of triple negative breast cancer (TNBC) continues to represent a significant challenge. Cytotoxic chemotherapies that often include doxorubicin (Adriamycin) remain the standard of care in breast cancer; while generally successful, patients frequently relapse with therapy resistant disease. Immunotherapy has emerged as a treatment modality with great promise, but has seen limited success in breast cancer therapy due, in large part, to the low immunogenicity of breast cancer cells. In view of the fact that doxorubicin has been shown to improve tumor immunogenicity by promoting the secretion of damage-associated molecular pattern (DAMP) molecules and immunogenic cell death (ICD), our recent goals were to enhance sensitivity to chemotherapy by both cell autonomous and cell non-autonomous strategies.**

Tumor cells reduce their immunogenicity in part through epigenetic mechanisms. The epigenetic regulator and chromatin remodeling complex (CRC), the nucleosome remodeling factor (NURF), suppresses tumor cell antigenicity and as a result inhibits T-cell–mediated antitumor activity NURF depletion from mouse models of TNBC enhances tumor cell immunogenicity. In efforts towards developing a novel and more effective therapy for TNBC, we combined NURF depletion with doxorubicin, a standard of care cytotoxic chemotherapy that targets topoisomerase II (Topo II). NURF-depleted cells demonstrate enhanced sensitivity to doxorubicin *in vitro* including increased DNA damage, reduced cell division, and suppressed proliferative recovery (cell autonomous effects). We further observe increased tumor cell immunogenicity, as demonstrated by increased sensitivity to the cytotoxic activities of natural killer (NK) cells *in vitro* and an enhanced antitumor response *in vivo*, which we postulate is related to the promotion of autophagy. In addition, we are in the process of testing pharmacological inhibitors of NURF in combination with cancer chemotherapeutic drugs with the goals of ultimately translating these findings to the clinic.

15. SUBJECT TERMS
Autophagy; tumor relapse; chemotherapy; immunotherapy; NURF

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1. **INTRODUCTION:** The objective of this project is to understand the role of autophagy in chemotherapy induced breast tumor dormancy and disease recurrence.

2. **KEYWORDS:** tumor dormancy; disease relapse, chemotherapy, autophagy, immunotherapy

3. **ACCOMPLISHMENTS:**

What were the major goals of the project?

The major goals of this project were to understand the role of autophagy in chemotherapy induced tumor dormancy and to further understand the role of tumor interferon gamma in determining disease recurrence under immune pressure.

What was accomplished under these goals?

I apologize for not providing the appropriate clarifications for essentail modifications to the proposed studies presented in the original statement of work (SOW). To address these oversights, I have indicted the specific experimental approaches taken from the SOW for the partnering PI and how the work we have performed is consistent with these approaches.

Two critical points to emphasize.

1. *Use of the 4T1 breast tumor cell line as opposed to the MMC cells.* The 4T1 cell line is, like the MMC cells, a murine breast tumor cell line, which allows for experiments to be performed in both immune competent and immune deficient animals. Our decision to switch to these cells is because they are well-characterized in the literature as a model of triple negative breast cancer (TNBC), a type of breast cancer that is particularly difficult to treat effectively, as the disease often recurs after therapy. Furthermore, these cells have been developed by Dr. Joseph Landry (who has assisted with this project) with knockdown of NURF, the epigenetic regulator that suppresses the immune response. Given the relatively low immunogenicity of breast cancer, this work allows us to develop a strategy for sensitization to chemotherapy (doxorubicin or Adriamycin (ADR) and recently paclitaxel) that has very high clinical potential for improving breast cancer therapeutic response. We should note that our recent grant submission to the DOD Breast Cancer Research program on this topic received an outstanding score (1.2).

2. *Response of NK cells and T cells to chemotherapy-treated breast tumor cells.* The original SOW focused on interferon gamma response to breast tumor cells exposed to chemotherapy. Interferon gamma is generated by NK cells and T cells (among other cell types such as macrophages). We are using NK cell killing and T cell response and killing as surrogates for interferon gamma activity.

Below are listed the components of the SOW that were/are the responsibility of the Partnering PI, and the accomplishments related to these components.

1.1. In vitro assays for the induction and evaluation of immunogenic and non-immunogenic apoptosis, autophagy and senescence in MMC and SKBR3 tumor cell lines.

We determined that ADR, but not radiation, induced what appears to be a non-immunogenic form of autophagy in the 4T1 cells and the non-immunogenic form switched to an immunogenic form of autophagy in the 4T1 cells after NURF depletion (Figure). These results were supported by experiments using a NURF small molecule inhibitor (AU1) (Fig. 4).

1.2. In vivo studies in FVBN202 mice challenged with MMC tumor cells, immunogenic and non-immunogenic chemotherapy.

Sensitivity to chemotherapy was enhanced as determined by in vivo studies in BALB/c mice challenged with 4T1 breast tumor cells where NURF was depleted, consistent with the premise that immunogenicity was enhanced. These are the equivalent of FVBN202 mice challenged with chemotherapy treated MMC cells, and are the result of switching from MMC to the 4T1 breast cancer model. These results were determined based on a series of in vivo studies in immunocompetent BALB/c and compromised NSG mouse models (see Fig. 1 and 7).

1.3. Evaluate caspase-dependent and caspase-independent apoptosis by the chemotherapeutic drugs ADR, MTX and RM in the presence or absence of the caspase inhibitor Z-VAD

We observed that apoptosis was increased when 4T1 breast tumor cells with knockdown of the epigenetic regulator NURF were exposed to ADR (Fig 2). We are in the process of inhibiting apoptosis to determine if modulation of apoptosis alters sensitivity to ADR.

Tumor challenge studies in FVBN202 mice bearing MMC tumors, chemotherapy with or without blockade of autophagy in vivo (Partnering PI: chemotherapy and chloroquine treatment).

We demonstrated that a blockade to autophagy suppressed the immunogenic response to chemotherapy. These results were determined based on a series of in vivo studies in immunocompetent BALB/c and compromised NSG mouse models (see Fig. 1 and 7).

2.1 - Determine immunogenic and non-immunogenic autophagy and apoptosis in tumor lesions by IHC

We have tumors in blocks from our in vivo treatment of 4T1 wild type cells and NURF depleted cells with ADR. Studies are currently in progress to evaluate autophagy and apoptosis in these tissues.

2.2 Generate Atg5, Atg12 and/or Beclin knocked down MMC and SKBR3 cells

4T1 wild type and NURF depleted cells where autophagy was silenced by ATG5 KD were generated and characterized both in vivo and in vitro for ADR induced immunogenicity. The results of these studies show that enhanced immunogenicity with ADR treatment of NURF depleted cells requires autophagy (is dependent on ATG5) (see Fig 7). Studies are in progress to generate PyMT cells with NURF knockdown and autophagy silencing. PyMT are another generally accepted model of triple negative breast cancer that we are using to substantiate our findings in the 4T1 cells.

2.3. In vitro studies on the effect of IFN-γ in the retention of autophagy in MMC or SKBR3 tumor cell lines in the presence or absence of ADR, MTX or RP

We demonstrate that Natural Killer cell activity is increased against NURF knockdown cells exposed to ADR, indicative of increased generation of interferon gamma (Fig. 1C). We also show that this effect depends, in part, on autophagy, using our 4T1 ATG5 KO cells (Fig 7C). NK cells are the major tumor resident immune cells that generate interferon gamma, which has direct antitumor effects including reduced tumor cell proliferation, increased tumor cell immunogenicity, and induced tumor cell apoptosis. Studies are in progress to make similar assessments in the PyMT cells.

Analysis of immunogenic and non-immunogenic autophagy

This was addressed in sections 1.1 and 2.1.

2.4. Chemotherapy combined with blockade of autophagy in FVBN202 mice challenged with MMC IFN-γ Rα low tumor cells (Partnering PI: chemotherapy and chloroquine)

4T1 cells where autophagy was genetically silenced were generated, as above. Studies are in progress to generate PyMT cells with autophagy silencing. We are in the process of creating the IFN-γ Rα low cells using CRISPR/Cas9 to test its effects on ATG5 KD NURF depleted tumor growth in vivo.

-Perform confirmatory tumor challenge experiments in FVB mice using Atg5, Atg12 and/or Beclin knocked down MMC IFN-γ Rα low tumor cells.

Studies are planned/in progress to deplete the IFN-g Ra in the ATG6 NURF KD 4T1 cells and repeat in vivo studies as shown ion Fig 7.

- Parallel studies using SKBR3 cells expressing IFN-γ Rα low grown in NOD/SCID/gamma chain null (NSG) mice in the presence or absence of chemotherapy and blockade of autophagy and IFN-γ. (Partnering PI/Initiating PI)

As indicated above, studies are planned/in progress to deplete select components of the immune system that generate IFN-γ (T cells, NK cells and macrophages) to determine which cells are critical for mediating the immune response to cancer therapy.

Study Results

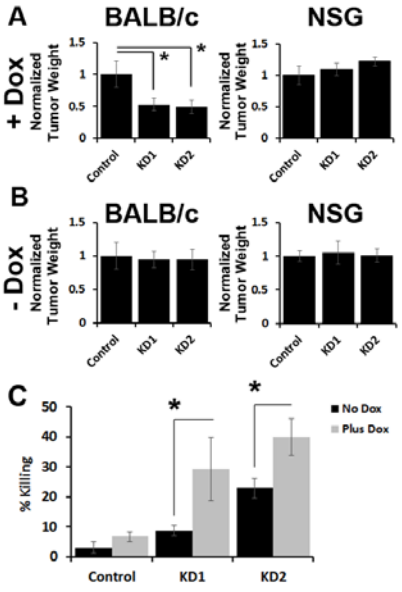

Fig. 1 ADR Enhances Antitumor Immunity to NURF Depleted Tumors. (A-B) WT (expressing a control shRNA) or BPTF KD (KD1 and KD2) 4T1 cells were inoculated into BALB/cJ or NSG mice and treated with **(A)** ADR 5mg/kg once a week for 3 weeks or a **(B)** PBS control. Tumors weighed 3 weeks (-Dox) or 4 weeks (+Dox) post inoculation. Normalized tumor weights are shown. Two-tailed t-test p values *=<0.05. SD error bars, N>6 tumors/group. **(C)** WT or BPTF KD 4T1 cells were treated with 50 nM ADR for 48 hours prior to coculture with mouse NK cells at an effector:target ratio of 10:1. Target cell killing was measured 24 hours after initial coculture by LDH release. Two-tailed t-test p values *=<0.05. SD error bars, N=3 replicates.

During the course of the past year, to complete the SOW for this project, we initiated studies to determine whether silencing of an epigenetic inhibitor and chromatin remodeling complex, specifically, the nucleosome remodeling factor NURF, could enhance the response of breast tumor cells to therapy through both cell autonomous (tumor cell direct) and cell non-autonomous (immune system based) mechanisms. In mammals, NURF has 3 subunits: the essential and largest subunit bromodomain PHD-finger transcription factor (BPTF). Knockout of BPTF achieves specific depletion of NURF because BPTF is exclusive to NURF, and it is required for its function (1,2).

To test whether NURF depletion would improve the immunogenicity of breast tumors responding to ADR, we implanted BALB/cJ or NSG mice with wild type (WT; expressing a control shRNA) or BPTF KD (knockdown) 4T1 cells and treated the mice with ADR (5 mg/kg once a week for 3 weeks). This dose of ADR promotes tumor immunogenicity, but does not itself suppress tumor growth (3). Comparing the right and left panels of **Fig. 1A,** we observed an enhanced antitumor effect of ADR to BPTF KD cells in immune competent BALB/c mice, but not immune compromised NSG mice, supporting our hypothesis that NURF inhibition would enhance

chemotherapy induced tumor immunogenicity. However, unlike the case in the 67NR and 66cl4 cells (not shown), BPTF KD alone does not result in suppression of 4T1 breast tumor growth (left panel of **Fig. 1B**). This is because to observe the enhanced antitumor immune response to NURF depleted 4T1 tumors, immune suppressive cells must be depleted (4). This, in fact, provides an advantage to experiments performed with 4T1 cells in tumor-bearing animals in that immune stimulation by ADR can be distinguished from the immune stimulatory effects of BPTF KD. In further support of the premise that NURF depletion enhances the therapy induced antitumor immune response, we do observe an enhanced NK cell cytolytic activity to ADR treated BPTF KD 4T1 cells compared to WT (control shRNA expressing) when co-cultured *in vitro* (**Fig. 1C**).

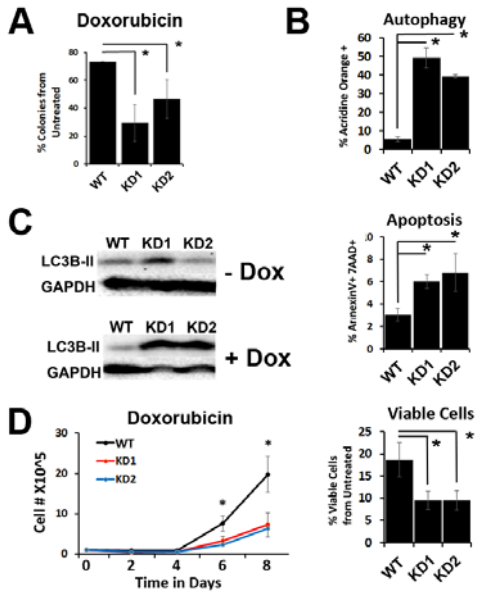

Fig. 2. BPTF Depleted 4T1 Cells Enter Autophagy After ADR Treatment. (A) Measurement of WT (control shRNA expressing) and BPTF KD (KD1 and KD2) 4T1 cell sensitization to 50 nM 24 hr ADR exposure by clonogenic survival. Percent of surviving cells are shown compared to untreated cells. **(B)** WT or BPTF KD 4T1 cells were treated with 50 nM ADR for 48 hours and flow cytometry was used to measure autophagy by acridine orange staining, apoptosis by annexin V staining and viable cells by trypan blue exclusion. **(C)** Western blotting for LC3BII from WT or BPTF KD 4T1 cells treated with 50 nM ADR for 48 hours. GAPDH was used as a loading control. **(D)** WT and BPTF KD 4T1 cells were treated with 50 nM ADR (Day 0) for 2 days, followed by recovery in growth media for 6 days. Viable cells were counted every 2 days. All panels are average, or representative, of 3 biological replicates.

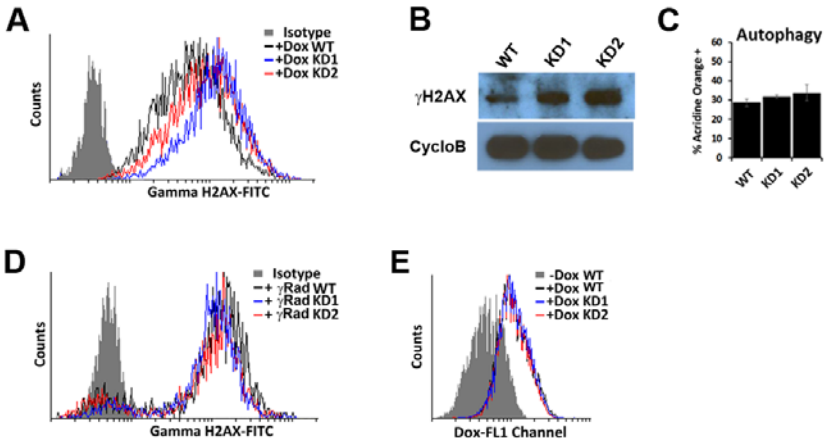

Fig. 3 BPTF Depleted 4T1 Cells Incur ADR Induced DNA Damage. (A) γH2AX was measured by flow cytometry after WT (control shRNA expressing) and BPTF KD (KD1 and KD2) 4T1 cells were treated with 50 nM ADR for 48 hours. **(B)** Western blotting of γH2AX from WT and BPTF KD cells treated with 50 nM ADR for 48 hours. Cyclophillin B was used as a loading control. **(C)** Autophagy was measured by acridine orange staining 48 hours after WT and BPTF KD 4T1 cells were treated with 5 Gy γ-radiation exposure. **(D)** γH2AX was measured by flow cytometry 30 minutes after WT and BPTF KD 4T1 cells were treated with 6Gy γ-radiation. **(E)** Flow cytometry measurement of 1 ug/ml ADR accumulation after 24 hours exposure to WT and BPTF KD 4T1 cells. Each panel representative of 3 biological replicates.

We hypothesized that the observed enhancement of antitumor responses in BPTF KD cells exposed to ADR could be derived from two sources: (i) non-cell autonomous effects, specifically the generation of damage-associated molecular pattern (DAMP) molecules and immunogenic cell death (ICD) that enhance tumor cell immunogenicity and (ii) cell autonomous effects, specifically an increase in DNA damage via facilitation of the accessibility of DNA to the enzyme, Topoisomerase II. We further propose that the immunogenicity could be a consequence of chemotherapy-induced autophagy in the BPTF KD cells.

To test these hypotheses, we first asked whether BPTF KD could enhance ADR sensitivity in cell culture (i.e., a cell autonomous effect). These studies demonstrated reduced clonogenic survival of

BPTF KD 4T1 cells compared to WT cells (expressing a control shRNA) when exposed to ADR (**Fig. 2A**). The reduced growth of ADR-treated BPTF KD 4T1 cells (between 40-60% reduction) coincides with a large increase (8-10 fold) in autophagy (initial studies based on flow analysis of acridine orange staining) and a smaller increase (~2 fold) in apoptosis (annexin V + 7AAD staining)(**Fig. 2B**). Consistent with these observations, BPTF KD results in increased LC3BII conversion indicative of the promotion of autophagy (**Figure 2C**), and delayed proliferative recovery after exposure to ADR (**Fig. 2D**).

ADR is known to induce DNA damage by poisoning Topoisomerase II (5). Using γH2AX levels as our endpoint, we observed increased DNA damage in ADR treated BPTF KD 4T1 cells relative to WT cells (expressing a control shRNA) by flow cytometry and Western blotting, suggesting enhanced Topo II poisoning (**Fig. 3A,B**). As a further control, we observed similar levels of autophagy and γH2AX in BPTF KD cells treated with γ-radiation, a DNA damaging agent independent of Topo II (**Fig. 3C,D**) (6) to which ***cells are not sensitized by NURF depletion*** (**see Fig. 5).** The enhancement of ADR sensitivity to BPTF KD cells is not simply a consequence of greater cellular drug accumulation, because we observe equivalent ADR accumulation into BPTF KD cells by flow cytometry (**Fig. 3E**).

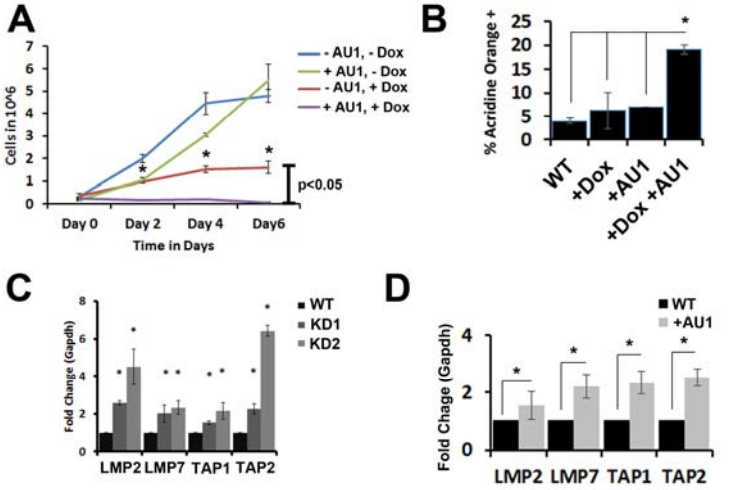

Fig. 4. BPTF Small Molecule Inhibitor AU1 Enhances Sensitivity to ADR and Improves Antigenicity. (A) WT 4T1 cells were pretreated with 10 μM AU1 for 2 days (Day -2) then treated with 50 nM ADR for 2 days, followed by recovery in growth media for 4 days. Viable cells were counted every 2 days. **(B)** WT 4T1 cells were pretreated with 10 μM AU1 for 2 days then treated with 50 nM ADR for 48 hours and flow cytometry was used to measure autophagy by acridine orange staining. **(C)** Lmp2, Lmp7, Tap1, and Tap2 expression measured from total RNA by qRT-PCR using Gapdh normalization from WT or BPTF KD 4T1 cells. **(D)** 4T1 cells were treated with 10 μM of the BPTF inhibitor AU1 for 12 days. Lmp2, Lmp7, Tap1, and Tap2 expression measured from total RNA by qRT-PCR using Gapdh normalization. Two-tailed t-test p values *=<0.05. SD error bars N=3 biological replicates.

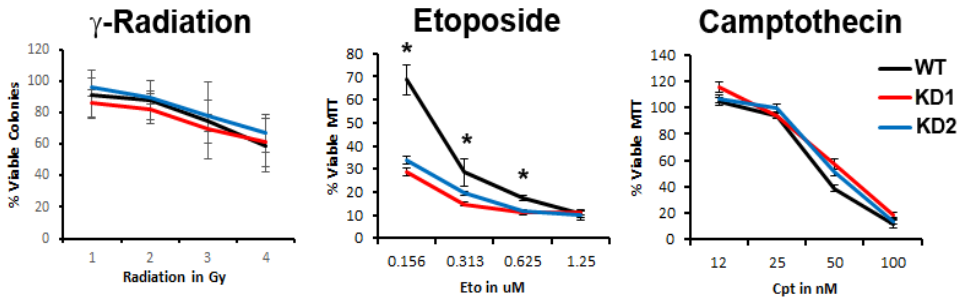

Fig. 5. BPTF Depletion Sensitizes Breast Cancer Cells to Other Chemotherapies. The effects of γ-radiation, etoposide, or camptothecin exposure on WT (control shRNA expressing) or BPTF KD (KD1 or KD2) 4T1 cell growth was determined by MTT assay (chemotherapies) or clonogenic survival (radiation). N=3 Replicates, *TTest p=0.05.

Figure 4 indicates that the reduced growth and enhanced autophagy observed in ADR treated BPTF KD 4T1 cells is also observed when WT 4T1 cells are pretreated with the NURF pharmacologic inhibitor AU1 (**Fig. 4A,B**) (7). This is a critical finding if we ultimately aspire to using pharmacological NURF inhibitors to enhance chemo-sensitivity in TNBC. Consistent with what we showed previously for BPTF KD 4T1 cells (4), AU1 treatment activates antigen

processing and presentation genes *Lmp2*, *Lmp7*, *Tap1*, and *Tap2* that have key roles in regulating tumor cell antigenicity (**Fig. 4C**). These data suggest that NURF can be pharmacologically targeted for therapeutic benefit.

We also asked whether enhanced sensitization to other chemotherapeutic agents (and ionizing radiation) might be observed with BPTF KD cells, potentially broadening the implications of our finding. To this end, we examined sensitivity to γ-radiation by clonogenic survival assay and to etoposide, and camptothecin using the standard MTT assay. We found that BPTF KD enhanced 4T1 breast tumor cell sensitivity to the Topo II poison etoposide, but not to the Topo I poison camptothecin or γ-radiation (**Fig. 5**). The selective enhancement of ADR and etoposide activity to NURF-depleted cells over camptothecin or radiation (**Fig. 5**) suggests functional connections between NURF and Topo II. ***However, it is critical to recognize that the lack of sensitization in cell culture does not exclude the possibility that an enhanced immune response might become evident in vivo through cell non-autonomous actions (e.g., DAMP secretion (8) mediated by autophagy).*** We plan on assessing the extent of autophagy induction to guide our decision as to which drugs to test *in vivo* as well as based on whether autophagy inhibition interferes with sensitization to ADR in tumor-bearing animals.

A seminal study by Michaud et al. has argued for the premise that the effectiveness of chemotherapy in cancer requires immune activation through the autophagy-mediated secretion of DAMPs (9). **Figure 2C** presents data indicating that the extent of autophagy is increased by 8- to 10-fold in ADR-treated BPTF KD cells compared to WT cells. These findings provide an opportunity to investigate the contribution of autophagy and autophagy-mediated secretion of DAMPS to chemo-sensitivity of breast tumor cells (8, 10). These studies are also relevant to the current clinical efforts to sensitize malignancies to chemotherapy and radiation via autophagy inhibition (11). If, in fact, autophagy inhibition i*nterferes* with tumor cell immunogenicity rather than the sensitization that is often observed in cell culture and in xenograft models (where, of course, the immune system involvement is thought to be minimal), then the strategy of autophagy inhibition could prove to be highly counterproductive. These findings will also allow us to define the cell non-autonomous impact of NURF depletion on autophagy and immune activation.

We have demonstrated that ADR selectively enhances autophagy in NURF-depleted cells and that NURF-depleted cells appear to have increased immunogenicity both in cell culture and in tumor-bearing animals. We further show enhanced sensitivity to ADR in cell culture as a cell autonomous effect that may be a consequence of increased DNA damage. A central question that remains is the nature of the chemotherapy-induced autophagy that improves breast tumor cell immunogenicity. The most common form of autophagy is cytoprotective in that its inhibition confers chemosensitivity or radiosensitivity (12). The cytotoxic form either directly promotes cell death (although this point is admittedly controversial) or is permissive for apoptosis (13). The cytostatic form mediates growth arrest (and cells recover proliferative function when it is inhibited). Finally, and most relevant to the current work, the nonprotective form is identified by the absence of any change in chemosensitivity or radiosensitivity when it is inhibited (12). In preliminary studies (**Fig. 6**) we demonstrate that autophagy inhibition by chloroquine fails to alter sensitivity to ADR in the 4T1 breast tumor cell line. This finding is consistent with previous work by our laboratory and others demonstrating that both ionizing radiation and cisplatin promote nonprotective autophagy in 4T1

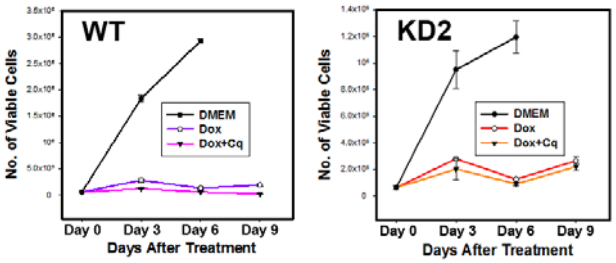

Fig. 6. ADR Induced Autophagy to BPTF Depleted Cells is Nonprotective. WT (control shRNA expressing) and BPTF KD (KD2) 4T1 cells were treated with 50 nM ADR (Dox) with or without 5mM Chloroquine (Cq) (Day 0) for 2 days, followed by recovery in growth media for 9 days. Viable cells were counted every 2 days. Panels are average, or representative, of 3 biological replicates.

breast tumor cells (14-16). Furthermore, the cell culture studies by Michaud et al (9) that reported the immune stimulatory effects of autophagy in fact identified nonprotective autophagy in that ATG5 knockdown in cell culture did not sensitize the tumor cells to mitoxantrone or oxaliplatin (two DAMP and ICD inducing chemotherapies).

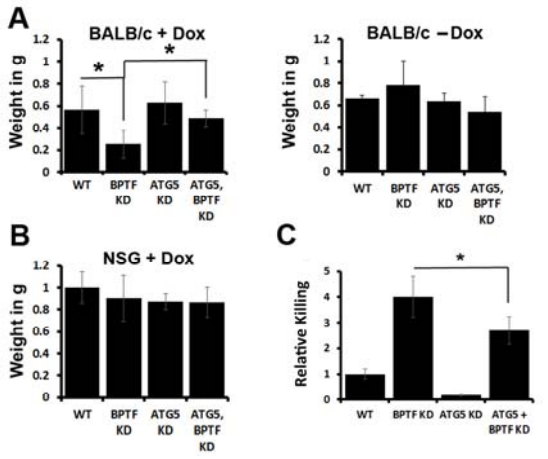

Fig 7) Autophagy is Required for the Antitumor Immune Response to NURF Depleted Tumors. (A-B) WT (expressing a control shRNA), BPTF KD, ATG5 KD or BPTF + ATG5 KD 4T1 cells were inoculated into BALB/cJ mice and treated with **(A)** doxorubicin 5mg/kg once a week for 2 weeks or a **(B)** PBS control. **(C)** Same experiment as above except NSG mice were used and treated with 5mg/kg once a week for 2 weeks. Tumors from A, B were weighed 4 weeks post inoculation. Two-tailed t-test p values *=<0.05. SD error bars, N>4 tumors/group. **(C)** WT (expressing a control shRNA), BPTF KD, ATG5 KD or BPTF + ATG5 KD 4T1 cells 4T1 cells were treated with 50 nM doxorubicin for 48 hours prior to coculture with mouse NK cells at an effector:target ratio of 10:1. Target cell killing was measured 24 hours after initial coculture by LDH release and expressed relative to WT. Two-tailed t-test p values *=<0.05. SD error bars, Representative of N=3 replicates.

Our findings from this award currently appear to be consistent with the hypothesis proposed by Michaud et al. (9) as the enhanced antitumor effects of doxorubicin to BPTF KD 4T1 tumors in immune competent BALB/c mice are eliminated when autophagy is inhibited by ATG5 shRNA silencing **(Fig. 7A)**. Furthermore, enhanced antitumor activity of doxorubicin to BPTF KD tumors was not observed in NSG mice demonstrating that the immune component is critical for the effect **(Fig. 7B)**. The importance of autophagy for the antitumor immune response to doxorubicin treated tumors correlates with its importance for NK cell mediated killing in vitro **(Fig 7C)**.

Summary and Overview Tumor cells reduce their immunogenicity in part through epigenetic mechanisms. Targeting these mechanisms (a cell non-autonomous approach) would represent a therapeutically viable strategy to reestablish antitumor immunity because, unlike genetic changes, which are permanent, epigenetic changes are reversible and are catalyzed by "druggable" enzymes. We have recently discovered that the epigenetic regulator and chromatin remodeling complex (CRC), the nucleosome remodeling factor (NURF), suppresses tumor cell antigenicity. NURF depletion from mouse models of TNBC enhances tumor cell immunogenicity, which we have shown can lead to complete regression in our metastatic breast cancer tumor models.

In efforts towards developing a novel and more effective therapy for TNBC, we combined NURF depletion with ADR, a standard of care cytotoxic chemotherapy that targets topoisomerase II (Topo II). NURF-depleted cells demonstrate enhanced sensitivity to ADR *in vitro* including increased DNA damage, reduced cell division, and suppressed proliferative recovery (cell autonomous effects). We further observe increased tumor cell immunogenicity, as demonstrated by increased sensitivity to the cytotoxic activities of natural killer (NK) cells *in vitro* and an enhanced antitumor response *in vivo*. Given the ongoing development of pharmacological agents that can target NURF, we believe that these findings can ultimately be translated to the clinic to improve the response of (triple negative) breast cancer to therapy.

Relevance and Implications Despite advances in cancer therapeutics over recent decades, developing effective strategies for the treatment of triple negative breast cancer (TNBC) continues to represent a significant challenge. Cytotoxic chemotherapies that often include ADR remain the standard of care in breast cancer; while generally successful, patients frequently relapse with therapy resistant disease. Immunotherapy has emerged as a treatment modality with great promise, but has seen limited success in breast cancer therapy due, in large part, to the low immunogenicity of breast cancer cells. In view of the fact that ADR has been shown to improve tumor immunogenicity by promoting the secretion of damage-associated molecular pattern (DAMP) molecules and immunogenic cell death (ICD), we have developed a unique strategy to enhance sensitivity to chemotherapy by both cell autonomous and cell non-autonomous mechanisms.

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What opportunities for training and professional development has the project provided?

The project provided for the training and professional development of Dr. Theresa Thekkudan and Ms. Liliya Tyutyunyk-Massey, a PhD candidate in Dr. Gewirtz's laboratory.

How were the results disseminated to communities of interest?

The results have been disseminated in poster presentations at local (Massey Cancer Center, Virginia Commonwealth University) and national (American Association for Cancer Research) scientific meetings and in published papers.

What do you plan to do during the next reporting period to accomplish the goals?

With regard to specific tasks indicated in the SOW that have not been completed.

1. We have tumors in blocks from our in vivo treatment of 4T1 wild type cells and NURF depleted cells with ADR. Studies are currently in progress to evaluate autophagy and apoptosis in these tissues.
2. Studies are in progress to generate PyMT cells with autophagy silencing.
3. Studies are in progress to assess Natural Killer cell activity against PyMT cells with autophagy silencing and exposed to ADR, indicative of the generation of interferon gamma.
4. Studies are planned/in progress to deplete select components of the immune system that generate IFN-γ (T cells, NK cells and macrophages) to determine which cells are critical for mediating the immune response to cancer therapy.

Our preliminary studies utilized primarily acridine orange staining as a quantitative indication of autophagy. However, recognizing the limitations of this approach we will confirm that ADR preferentially promotes autophagy in BPTF KD 4T1 cells (two different shRNA BPTF KDs) during exposure and recovery (for 8-12 days) from 50 nM ADR for 48 hours (chronic exposure conditions that are pharmacologically relevant to tumors in treated patients) (10, 17) . For determination of autophagic flux, the completion of the autophagic process, assays will include degradation of p62/SQSTM1 and conversion of LC3I to LC3II in the absence or presence of chloroquine (18). Cells will be transfected with tandem RFP-GFP-LC3B to distinguish between early and late stages of autophagy; co-localization of LC3 with LAMP will assess autophagosome/lysosome fusion (18). We will further assess apoptosis and overall cell death by Caspase 3 cleavage and annexin V/PI staining, respectively, although we expect minimal apoptosis as was the case in the 4T1 cells. Instead, it is likely that the cells are undergoing senescence arrest in tandem with autophagy (19). Guided by our preliminary data , we will monitor γH2AX by flow cytometry and Western blotting as a measure of DNA damage.

Genetic and pharmacological approaches will be utilized to identify the functional form of the autophagy. Genetic inhibition will be achieved by ATG5, ATG7, or Beclin1 shRNA KD (18). Independently, chloroquine or bafilomycin A1 will be used to inhibit late stage autophagy (18). We anticipate that the autophagy will prove to be the nonprotective form, as strongly suggested by the data in **Figure 6**. This outcome would be consistent with the Michaud et al. findings relating to autophagy-enhanced immunogenicity (9), where genetic autophagy inhibition failed to increase the extent of apoptosis (20).

To determine the utility of inhibiting NURF activity pharmacologically, we will repeat a limited number of key experiments using the BPTF inhibitor AU1 (**see Fig. 4**) (7). Key experiments will include enhanced ADR sensitivity, induction of autophagy, identification of the functional form of autophagy, secretion of DAMPs after ADR exposure and associated immune cell (NK and/or T cell) cytolytic activity. BPTF KD cells will be used in these experiments to determine AU1 specificity (if AU1 is specific for BPTF, then little change should be observed to BPTF KD cells). These studies will provide proof-of-principle for ultimately translating this work to the clinic once the AU1 compound has been sufficiently tested for pharmacokinetic properties and toxicity. Completing this work will determine if the nature of ADR-induced autophagy is altered with BPTF KD, its consequences to cancer cell recovery from chemotherapy, and how it relates to cancer cell immunogenicity.

4. IMPACT:

What was the impact on the development of the principal discipline(s) of the project?

Salient findings:

NURF acts as a modulator of the immune response, either/or T cell or NK cell mediated, to breast tumor cells.
Knockdown of NURF enhances sensitivity to ADR by cell non-autonomous pathways which likely result in increased immunogenicity.
Knockdown of NURF enhances sensitivity to ADR by cell autonomous pathways. These may include increased DNA damage and the promotion of autophagy.
Knockdown of NURF may also enhance sensitivity to other chemotherapeutic drugs. Drugs we will specifically test are those which enhance tumor cell immunogenicity.
A pharmacologic inhibitor of NURF may also be effective in sensitizing breast tumor cells to chemotherapy. A NURF pharmacologic inhibitor is developed (AU1.

What was the impact on other disciplines?

These results have broad impacts to the field of **epigenetics** in that they characterize the nuclear functions for the NURF complex in chemosensitization, which has not been recognized previously. This work also has impact to the field of **immunotherapy,** strongly suggesting that inhibiting NURF is a novel means to enhance the immunogenicity of breast tumor cells, which has implications for the use of immunogenic modulators.

What was the impact on technology transfer?

None to report

What was the impact on society beyond science and technology?

None to report

5. CHANGES/PROBLEMS:

Changes in approach and reasons for change

Our current findings are now focused on Natural Killer cells and T cells, which generate interferon gamma to more broadly understand the nature of the immune response to therapy. We determined that focusing our studies exclusively on IFNγ was too limiting, as many cancer cells

can become IFNγ resistant as a means to adaption. We are also using 4T1 and PyMT breast tumor cells as models of triple negative breast cancer due to the difficulty of generating durable responses in the treatment of this form of the disease. Since we discovered that modulation of epigenetic regulation can enhance the sensitivity of breast cancer cells to doxorubicin (and recently paclitaxel), this appears to have potential to directly improve the treatment of breast cancer, particularly given the availability of a pharmacologic agent (AU1) that can simulate the silencing of NURF.

Actual or anticipated problems or delays and actions or plans to resolve them:

None to report.

Changes that had a significant impact on expenditures

None to report.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

None to report.

6. PRODUCTS:

- **Publications, conference papers, and presentations**

#3314 Synergistic effects of chemotherapy-induced autophagy and epigenetic remodeling. Liliya Tyutyunyk, Joseph Landry, Tareq Saleh, David Gewirtz. Virginia Commonwealth University, Richmond, VA.
Cytotoxic chemotherapy has been shown to enhance tumor cell immunogenicity by promoting the secretion of damage-associated molecular pattern (DAMP) molecules which, in turn, stimulates the immune response. These effects can be further stimulated by combining chemotherapy with an immunotherapy designed to enhance effector cell (CD8 T cell or NK cell) anti-tumor activity. Our studies demonstrate that knockdown of the epigenetic regulator NURF increases DNA damage (gamma H2AX staining) and autophagy (acridine orange staining) in breast tumor cells exposed to ADR and enhances growth inhibition as well as suppressing the capacity of the cells to recover and proliferate. Similar increases in autophagy were observed using a small molecule inhibitor of NURF,suggesting thatNURFcan be targeted therapeutically. Sensitization was not observed with ionizing radiation or cisplatin. Studies are in progress to assess the nature of autophagy in the NURF KD and NURF WT cells, distinguishing between the cytoprotective, cytotoxic, cytostatic and nonprotective forms. How NURF regulates ADR induced DNA damage is being investigated by mapping sites of damage genome wide. It is anticipated that enhanced cell autonomous tumor cell sensitivity in concert with improvements in tumor cell antigenicity (cell non-autonomous sensitization) achieved by NURFdepletion could improve anti-tumor immunogenicity, achieve tumor regression, reduce metastasis, and possibly achieve long term remission in breast cancer.
Proceedings of the American Association for Cancer Research Volume 58 April 2017

#5459 Autophagy and senescence as possible mechanisms leading to proliferative recovery and escape from treatment-induced tumor dormancy. Liliya Tyutyunyk, Theresa Thekkudan, Tareq Saleh, David A. Gewirtz. Virginia Commonwealth University, Richmond, VA.
Despite the ability of chemotherapy to eliminate the majority of tumor cells, some are able to

escape cell death and proliferate. In this study we evaluated mechanisms of autophagy and senescence, that in theory allow cells to escape apoptosis and might be responsible for cancer recurrence. Autophagy and senescence, either alone or in concert, may result in temporary growth arrest followed by proliferative recovery. With regard to autophagy, its function may be either cytoprotective, where autophagy inhibition results in sensitization to therapy or nonprotective, where autophagy inhibition does not alter sensitivity to chemotherapy and/or ionizing radiation. To determine the mode of autophagy in murine metastatic carcinoma 4T1 cell lines, autophagy was inhibited pharmacologically using chloroquine, and by genetic silencing of the Atg5 autophagy associated protein. Cells were treated with 1uM Adriamycin, stained with Acridine Orange to assess autophagy by fluorescent microscopy (autophagosomes appear orange-red) and autophagy quantified by Flow Cytometry. Induction and blockade of autophagy by chloroquine was confirmed by Western blotting for the appearance of LC3B and degradation of p62. Senescence was monitored by beta-galactosidase staining and quantified by Flow Cytometry based on C12FDG staining; senescence was confirmed based on the induction of p21 and p16. Adriamycin (ADR) exposure resulted in breast tumor cell death as well as prolonged growth arrest; some of the arrested cells eventually recovered and formed colonies. After exposure to ADR, 4T-1 cells were sorted based on intensity of beta-galactosidase staining and increased size (senescent cells experience changes in size and morphology). Beta--galactosidase positive cells were plated and monitored over time during which some of the cells recovered the capacity to proliferate. In separate studies with mouse mammary carcinoma cells, radiation also promoted cell death and prolonged growth arrest from which some cells were able to recover proliferative capacity. Our data suggests that although chemotherapy and radiation induce prolonged growth arrest and senescence, these features are not permanent and cells are able to escape and re-emerge from the senescent state to generate proliferating daughter cells. As pharmacologic inhibition of autophagy did not result in increased sensitivity to ADR in 4T1 cells (or to radiation in the mouse mammary tumor cells) or interfere with proliferative recovery, we postulate that inhibition of senescence associated pathways may block proliferative recovery and/or promote tumor cell killing in response to chemotherapy and/or radiation. Studies in progress are focused on modulation of c-myc and miR34 levels and the IL1/6/8 signaling axis as potential strategies for interference with senescence and suppression of residual (dormant) tumor growth and cancer recurrence.

Proceedings of the American Association for Cancer Research Volume 58 April 2017

Papers

Gewirtz, D, Alotaibi M, Yakovlev V, Povirk LF. Tumor cell recovery from senescence induced by radiation + PARP inhibition. Radiat Res. 2016 Oct;186(4):327-332. *These studies have suggested that the promotion of senescence by therapy does not result in a sustained growth arrest. These findings are highly relevant to the issue of tumor dormancy and cancer recurrence. The methods developed in these studies, particularly the sorting of senescent tumor cells are currently being extended to breast tumor cells (findings to appear in the final progress report).*

Gewirtz DA. The challenge of developing autophagy inhibition as a therapeutic strategy. Cancer Res. 2016 Oct 1;76(19):5610-5614. *This commentary addresses the efforts to sensitize different malignancies, including breast cancer, via the inhibition of autophagy, and the problems associated with this clinical strategy.*

Toma W, Kyte SL, Bagdas D, Alkhlaif Y, Alsharari SD, Lichtman AH, Chen ZJ, Del Fabbro E, Bigbee JW, Gewirtz DA, Damaj MI. Effects of paclitaxel on the development of neuropathy and affective behaviors in the mouse. Neuropharmacology. 2017 Feb 22;117:305-315. *This paper develops a novel strategy to mitigate the peripheral neuropathy induced by drugs utilized to treat breast, ovarian and lung cancer.*

Galuzzi et al. Molecular definitions of autophagy and related processes. EMBO J. EMBO J. 2017 Jul 3;36(13):1811-1836. Epub 2017 Jun 8. Review. *This review attempts to integrate the literature relating to autophagy.*

Presentations

- **Website(s) or other Internet site(s)**
- **Technologies or techniques**
- **Inventions, patent applications, and/or licenses**
- **Other Products**

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Dr. David Gewirtz (3 years) - supported by Virginia Commonwealth University and this contract.
Ms. Liliya Tyutyunyk-Massey (18 months)- supported by Virginia Commonwealth University and this contract.
Mr. Tareq Saleh (12 months) – supported by the Kingdom of Jordan
Dr. Joseph Landry (18 months)- supported by Virginia Commonwealth University

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Yes. Awarded Grant: National Institutes of Health Grant 1R01CA206028-01. Grant Period 3/01/2016 – 02/28/21. Co PI with Dr. Imad Damaj. 10% Effort. $280,293/annum. Mitigation of chemotherapy induced peripheral neuropathy. This project involves the utilization of nicotine and silent agonists of nicotine to suppress and/or prevent peripheral neuropathy induced by agents such as the taxanes and platinum based compounds utilized in the treatment of ***breast cancer***, ovarian cancer and lung cancer.

What other organizations were involved as partners?

None

8. SPECIAL REPORTING REQUIREMENTS:

COLLABORATIVE AWARDS:

9. APPENDICES: Published papers attached.

Radiosensitization by PARP Inhibition in DNA Repair Proficient and Deficient Tumor Cells: Proliferative Recovery in Senescent Cells

Author(s): Moureq Alotaibi , Khushboo Sharma , Tareq Saleh , Lawrence F. Povirk , Eric A. Hendrickson and David A. Gewirtz

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Radiosensitization by PARP Inhibition in DNA Repair Proficient and Deficient Tumor Cells: Proliferative Recovery in Senescent Cells

Moureq Alotaibi,[a,c] Khushboo Sharma,[a] Tareq Saleh,[a] Lawrence F. Povirk,[a] Eric A. Hendrickson[d] and David A. Gewirtz[a,b,1]

[a] *Department of Pharmacology and Toxicology, School of Medicine and* [b] *Massey Cancer Center, Virginia Commonwealth University, Richmond, Virginia, 23298;* [c] *Department of Pharmacology and Toxicology, College of Pharmacy, King Saud University, Riyadh, 11451, Kingdom of Saudi Arabia; and* [d] *Department of Biochemistry, Molecular Biology and Biophysics, University of Minnesota, Minneapolis, Minnesota 55455*

Alotaibi, M., Sharma, K., Saleh, T., Povirk, L. F., Hendrickson, E. A. and Gewirtz, D. A. Radiosensitization by PARP Inhibition in DNA Repair Proficient and Deficient Tumor Cells: Proliferative Recovery in Senescent Cells. *Radiat. Res.* 185, 229–245 (2016).

Radiotherapy continues to be a primary modality in the treatment of cancer. In addition to promoting apoptosis, radiation-induced DNA damage can promote autophagy and senescence, both of which can theoretically function to prolong tumor survival. In this work, we tested the hypothesis that autophagy and/or senescence could be permissive for DNA repair, thereby facilitating tumor cell recovery from radiation-induced growth arrest and/or cell death. In addition, studies were designed to elucidate the involvement of autophagy and senescence in radiosensitization by PARP inhibitors and the re-emergence of a proliferating tumor cell population. In the context of this work, the relationship between radiation-induced autophagy and senescence was also determined. Studies were performed using DNA repair-proficient HCT116 colon carcinoma cells and a repair-deficient ligase IV$^{-/-}$ isogenic cell line. Exposure to radiation promoted a parallel induction of autophagy and senescence that was strongly correlated with the extent of persistent H2AX phosphorylation in both cell lines, however, inhibition of autophagy failed to suppress senescence, indicating that the two responses were dissociable. Exposure to radiation resulted in a transient arrest in the HCT116 cells while arrest was prolonged in the ligase IV$^{-/-}$ cells, however, both cell lines ultimately recovered proliferative function, which may reflect maintenance of DNA repair capacity. The PARP inhibitors, olaparib and niraparib, increased the extent of persistent DNA damage induced by radiation exposure as well as the extent of both autophagy and senescence. Neither cell line underwent significant apoptosis by radiation exposure alone or in the presence of the PARP inhibitors. Inhibition of autophagy failed to attenuate radiosensitization, indicating that autophagy was not involved in the action of the PARP inhibitors. As with radiation alone, despite sensitization by PARP inhibition, proliferative recovery was evident within a period of 10–20 days. While inhibition of DNA repair via PARP inhibition may initially sensitize tumor cells to radiation via the promotion of senescence, this strategy does not appear to interfere with proliferative recovery, which could ultimately contribute to disease recurrence.

INTRODUCTION

Radiotherapy is used along with other modalities such as surgery, chemotherapy and immunotherapy to either shrink tumors before surgery or eliminate surviving tumor cells after surgery. While ionizing radiation is ultimately cytotoxic by virtue of inducing DNA damage, specifically double-strand breaks (DSBs) (*1–3*), radiation treatment also elicits a complex ensemble of responses that can moderate its toxic effects. Among these responses, autophagy and senescence are particularly intriguing because they can contribute to tumor control through autophagic cell death (*4*) or persistent growth arrest (*5*), respectively, but can also antagonize apoptosis and thereby shelter a population of dormant cells that may later reinitiate tumor regrowth (*6–9*).

There is extensive evidence that radiation can promote autophagy (*10*). Autophagy can function as a pro-survival mechanism or as pro-death mechanism, depending on the agents used and the experimental systems. The relationship between autophagy and DNA repair is unclear, but several published studies have shown that autophagy might be involved in DNA repair in cells exposed to DNA-damaging agents (*11–15*).

It is also well established that various forms of stress, particularly exposure to DNA-damaging agents such as radiation, can promote senescence (*5, 16, 17*). While senescence has often been considered to be an irreversible form of growth arrest, it is long established that telomerase can be reactivated in cells undergoing replicative senes-

Editor's note. The online version of this article (DOI: 10.1667/RR14202.1) contains supplementary information that is available to all authorized users.

[1] Address for correspondence: Massey Cancer Center, Virginia Commonwealth University, 401 College St., Richmond, VA 23298; email: david.gewirtz@vcuhealth.org.

cence, ultimately leading to an immortalized replicating cell population (*18*). Furthermore, there is clear experimental evidence for reversibility of senescence under select experimental conditions (*19*).

As for DNA damage and senescence it has been established that ionizing radiation induces DNA damage foci, the majority of which are transient and disappear within hours after treatment (*20, 21*). While some foci may persist for months, the repair of DNA DSBs in senescent cells may result in recovery and regrowth. In fact, there is evidence that senescent cells can repopulate after exposure to chemotherapeutic agents and radiation (*16, 19, 22–24*).

From a clinical perspective, the possibility of sensitization to radiation (and chemotherapy) through the administration of PARP inhibitors to interfere with DNA repair continues to be an area of active inquiry (*25–28*). Interestingly, radiosensitization has been shown to lead primarily to an increase in senescence with minimal apoptosis (*29, 30*). Furthermore, the potential involvement of autophagy in radiation sensitization via PARP inhibition has not been investigated; this is relevant since it has been shown that autophagy and senescence may be closely associated responses in some studies (*31–33*).

The primary goal of this work was to understand the involvement of autophagy and senescence in the response to radiation-induced DNA damage, and the interplay between these responses and DNA repair. Our findings revealed that the extent of both autophagy and senescence correlates with the intensity of persistent unrepaired DNA damage. Furthermore, interference with DNA repair via PARP inhibition using olaparib (AZD-2281) or niraparib (MK-4827) may initially sensitize cells via increased autophagy and senescence, but not apoptosis. However, this strategy does not appear to interfere with proliferative recovery, which could, in theory, contribute to disease recurrence (*34–37*).

MATERIALS AND METHODS

Cell Line

HCT116 colon cancer cells were purchased from ATCC® (Gaithersburg, MD) and HCT116 ligase IV-deficient cells were generated as previously described elsewhere (*38*). HCT116 ligase IV-deficient and ligase IV-proficient cell lines were maintained as subconfluent cultures in RPMI 1640 media with 5% fetal bovine serum, 5% bovine calf serum, 2 m*M* L-glutamine and penicillin (Gibco®/Life Technologies, Gaithersburg, MD) and incubated at 37°C, 5% CO_2 in a humidified environment. In every experiment, cells were cultured under identical conditions and incubated overnight to allow for adherence prior to irradiation.

ATG5 and ATG7 silencing *shCon*, *shATG5* and *shATG7* plasmid constructs were isolated (Plasmid Midi Kit; QIAGEN®, Valencia, CA) using bacterial stocks (Sigma-Aldrich®, St. Louis, MO). Plasmid constructs were packaged into lentiviral particles using HEK 239T cells and a packaging mixture composed of Lipofectamine® (cat. no. 11669-019; Invitrogen™, Carlsbad, CA), psPAX2 and pMD2.G packaging constructs (nos. 12260, 12259; Addgene, Cambridge, MA). Growth media containing the viral particles was collected and used to infect HCT116 cells. Infected cells were then maintained with the selection marker, puromycin (2 μg/ml) throughout the course of the study.

Time Course of Radiation-Induced Effects on Cell Viability

Cells were plated in 6-well plates (generally 200,000 cells/well) and allowed to adhere overnight. The next day, cells were irradiated and the number of viable cells were counted at indicated time points for 5 days. In the case where a drug was co-administered (PARP inhibitors or autophagy inhibitors) with radiation treatment, cells were pretreated with the drug 3 h before irradiation and the drug was washed away 24 h postirradiation. In the case of the apoptosis inhibitor, Z-VAD-FMK, cells were pretreated for 3 h and maintained in the drug throughout the course of the study. At each time point, media was removed and cells were washed one time with 1X phosphate buffered saline (PBS). Trypsin (0.25%, 500 μl) was added to each well for harvesting and incubated for 5 min, then deactivated by 500 μl of fresh media to make up 1 ml of cell suspension. Cells were collected in 1 ml conical tubes (Eppendorf Inc., Westbury, NY) and 10 μl of cell suspension was added to 10 μl of trypan blue (0.4%), placed onto chamber slides of a hemocytometer (Hausser Scientific, Horsham, PA) and counted under a microscope.

Clonogenic Survival Assay

Cells (200) were plated in 6-well plates and allowed to adhere overnight. After 24 h, cells were pre-incubated with the indicated drug for 3 h and then exposed to the indicated dose of radiation. The following day, drug-containing media was removed, cells were washed and supplemented with fresh media that was replaced every other day for two weeks. On the day of staining, cells were fixed with 90% methanol for 10 min, and then stained with 1% crystal violet for another 10 min. Colonies were then washed with PBS three times to eliminate excessive crystal violet staining and counted manually.

Assessment of Autophagy by Acridine Orange Staining

Cells (50,000) were seeded in 6-well plates and allowed to adhere overnight, then exposed to radiation the following day. At the various time points, media was removed and cells washed once with 1X PBS. The acridine orange solution was made up in 1X PBS to a final concentration of 100 ng/ml in the dark and protected from light until ready for use. For flow cytometry, 10 μl of acridine orange solution was added to each sample and allowed to incubate for 15 min. Dye-containing media was then aspirated, plates were washed with 1X PBS and fresh media was added. Photographs were taken with an Olympus 1× 70 microscope and an Olympus SC 35 camera.

The cell population positively stained with acridine orange was quantified by flow cytometry. Treated cells were trypsinized, collected and centrifuged at 1,500 rpm for 5 min. Supernatant was removed and pellets were resuspended in 990 μl of 1X PBS. The cell suspension was filtered through a standard flow cytometry 40 micron filter (BD Falcon™; BD Biosciences, Bedford, MA). The acridine orange solution was made up in 1X PBS to a final concentration of 100 ng/ml in the dark and protected from light until ready for use. For flow cytometry, 10 μl of acridine orange solution was added to each sample and allowed to mix for 15 min. Acridine orange is excited at a wavelength of 525 n*M* for green fluorescence and 620 n*M* for red fluorescence.

Transfection of HCT116 Cells with RFP-LC3

The RFP-LC3 construct was generated by the Tolkovosky laboratory (*39*). HCT116 cells (1×10^6) were collected in a pellet, centrifuged and resuspended with the construct in 100 μl of the Amaxa® Nucleofector™ Kit V. One microgram of the RFP-LC3 vector was added to the suspension. The cell suspension was collected in a cuvette, then placed in nucleofector device to run program D-032. Media (500 μl) was added to the transfected cells and to transfer them

to a petri dish where cells were maintained under gentamicin (8 ng/ml) to maintain the stable transfection.

Cell Cycle Analysis

At the indicated time points, cells were trypsinized, collected and centrifuged at 1,500 rpm. The supernatant was aspirated, pellets washed in PBS and recentrifuged at 1,500 rpm. The supernatant was removed, 0.2 ml of PBS was added and pellets were gently mixed to form a single cell suspension. Cold 70% ethanol (1.8 ml) was gradually added into the cell suspension; cells were vortexed, centrifuged, ethanol was aspirated and cells were washed with PBS prior to addition of a staining solution [0.1% (v/v) Triton™ X-100 in 10 ml PBS, 2 mg of DNase free RNase A and 0.2 ml of the propidium iodide stock (1 mg/ml)] 2 h prior to flow cytometry.

Evaluation of Senescence by β-Galactosidase Staining

Beta-galactosidase staining was utilized as a marker of senescence. Cells were washed once with 1X PBS and fixed with 2% formaldehyde/0.2% glutaraldehyde for 5 min, washed again with PBS and finally incubated overnight in a staining solution composed of 1 mg/ml 5-bromo-4-chloro-3-indolyl-β-galactosidase in dimethylformamide (20 mg/ml stock), 5 m*M* potassium ferricyanide, 150 m*M* NaCl, 40 m*M* citric acid/sodium phosphate, 2 m*M* MgCl2, at pH 6.0 in CO2 at 37°C. The following day, cells were washed twice with PBS and images were taken.

For β-galactosidase detection by flow cytometry, cells were washed and incubated for 1 h in complete media containing 100 n*M* of bafilomycin A1 to induce lysosomal alkalinization. After incubation, C_{12}FDG working solution was added to each well for a final concentration of 33 μ*M*, and incubation was continued for another 1 h. Media was then aspirated and cells were washed twice with PBS. Cells were harvested, collected by centrifugation at 1,500 rpm, resuspended in PBS and analyzed by flow cytometry, as described above, but with excitation at 490 nm and a 514 nm emission filter. C_{12}FDG is hydrolyzed by upregulated β-galactosidase enzyme and becomes fluorescent at a wavelength of 500–510 n*M*.

Determination of γ-H2AX Intensity as a Marker of DNA Damage

Cells (5,000) were seeded in 4-chamber cover glass slides (Lab-Tek™ II) and allowed to adhere overnight. On the following day, cells were irradiated and fixed with 4% formaldehyde for 5 min at indicated time points. Cells were washed twice with 1X PBS, incubated at room temperature in 0.05% Triton X for 15 min, washed and incubated with 1X PBS containing 1% of BSA for 30 min to prevent nonspecific binding of the antibody. Finally, cells were incubated in a 1:10 dilution of γ-H2AX antibody (BD Pharmingen™, San Diego, CA) in 1% BSA for 1 h. Images were taken using an LSM 700 confocal microscope (Carl Zeiss MicroImaging Inc., Thornwood, NY).

Alternatively, for flow cytometry, cells were harvested at the indicated time points, fixed with 90% ethanol and maintained at −20°C until the day of the experiment. Cells were then centrifuged at 3,000 rpm for 5 min and resuspended in 1% BSA for 30 min. Gamma-H2AX antibody (BD Pharmingen) was added to the cells in a dilution of (1:200) and incubated at room temperature for 1 h. Cells were then analyzed by flow cytometry at an excitation wavelength of 488. Raw data were normalized according to the intensity of control samples (normalized mean intensity = intensity of the sample/the intensity of the corresponding control sample within the same experiment).

Evaluation of DNA Damage Extent by the Comet Assay

Cells (200,000) were plated in 6 cm dishes and treated as indicated. After 72 h, cells were gently scraped from the plates and 100,000 cells were mixed with molten LMAgarose (at 37°C) at a ratio of 1:10 (v/v). The mixture of cells and LMAgarose was then pipetted onto CometSlides™ (Trevigen® Inc., Gaithersburg, MD) and incubated for 30 min at 37°C. The slides were kept at 4°C for 10 min prior to being immersed in Lysis Solution (Trevigen) overnight. On the following day, the slides were immersed in 1X neutral electrophoresis buffer for 30 min at 4°C, set onto an electrophoresis tray for 45 min and electrophoresed at 1 volt per cm. Slides were then immersed in DNA precipitation solution (1 *M* ammonium acetate in 70% ethanol) for 30 min at room temperature, followed by 70% ethanol for another 30 min. Samples were dried at 37°C for 30 min and stained with the working dilution of SYBR® Green (Trevigen).

Evaluation of Apoptosis

After the indicated treatments, cells were harvested at the indicated time points and collected on a cytospin slide, fixed with formaldehyde (4%) for 5 min and washed with 1X PBS twice. Slides were fixed with acetic acid/ethanol (1:2) for 5 min and washed twice with 1X PBS. For the terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay, cells were blocked with BSA (1 mg/ml for 30 min) at room temperature, washed twice in PBS and incubated with enzyme mixture (terminal transferase, 25 m*M* $CoCl_2$, fluorescein-12dUTP) for 1 h at 37° to allow the enzymatic reaction. After washing with PBS, cells were stained with DAPI and images were taken using a fluorescence microscope.

For the PI/Annexin assay, adherent and non-adherent cells were harvested in Eppendorf tubes and pellets resuspended in 100 μl of binding buffer (BD Biosciences). Annexin-FITC (5 μl; BD Biosciences) and 5 μl of PI at 10 μg/ml (BD Biosciences) were added to the cell suspension and incubated for 15 min in the dark at room temperature. Annexin V binding buffer 1X (400 μl; BD Pharmingen) was added to each sample and samples were analyzed by flow cytometry at 530 n*M*.

Western Blotting

At the indicated time points, cells were harvested and mixed with lysis buffer (1 *M* Tris HCl, pH 6.8, 10% SDS) containing protease and phosphatase inhibitors (Sigma-Aldrich). Proteins were separated on 12% gels using SDS-PAGE and transferred onto nitrocellulose membranes. The membranes were blocked using nonfat dry milk and PBS for 30 min at room temperature, then incubated with the primary antibody overnight at 4°C. Primary antibodies used were as follows: anti-p62 (SQSTM1, cat. no. sc-28359; Santa Cruz Biotechnology®, Inc., Dallas, TX), anti-β actin (cat. no. sc-47778; Santa Cruz), anti-ATG5 (cat. no. 12994S; Cell Signaling Technology®, Danvers, MA), and anti-ATG7 (cat. no. 8558S; Cell Signaling). All primary antibodies were used at a 1:1,000 dilution. The following day, membranes were incubated with correspondent secondary antibodies for 1 h. Secondary antibodies used were goat anti-mouse IgG (Amersham, GE Healthcare Bio-Sciences, Pittsburgh, PA) and monkey anti-rabbit IgG (Amersham, GE Healthcare). Membranes were then washed three times and bands were detected using enhanced chemiluminescence detection reagents (Pierce™ Biotechnology, Rockford, IL).

Statistical Analysis

Statistical analyses were performed using StatView statistical software (SAS Institute Inc., Cary, NC). The data were expressed as means ± SE. Comparisons were made using two-way analysis of variance followed by the Bonferroni post hoc test. P values <0.05 were considered statistically significant.

RESULTS

Response of DNA Repair-Competent and DNA Repair-Deficient Cell Lines to Radiation Exposure

Radiation sensitivity in the HCT116 and the HCT116 ligase IV-deficient cell lines was determined by clonogenic

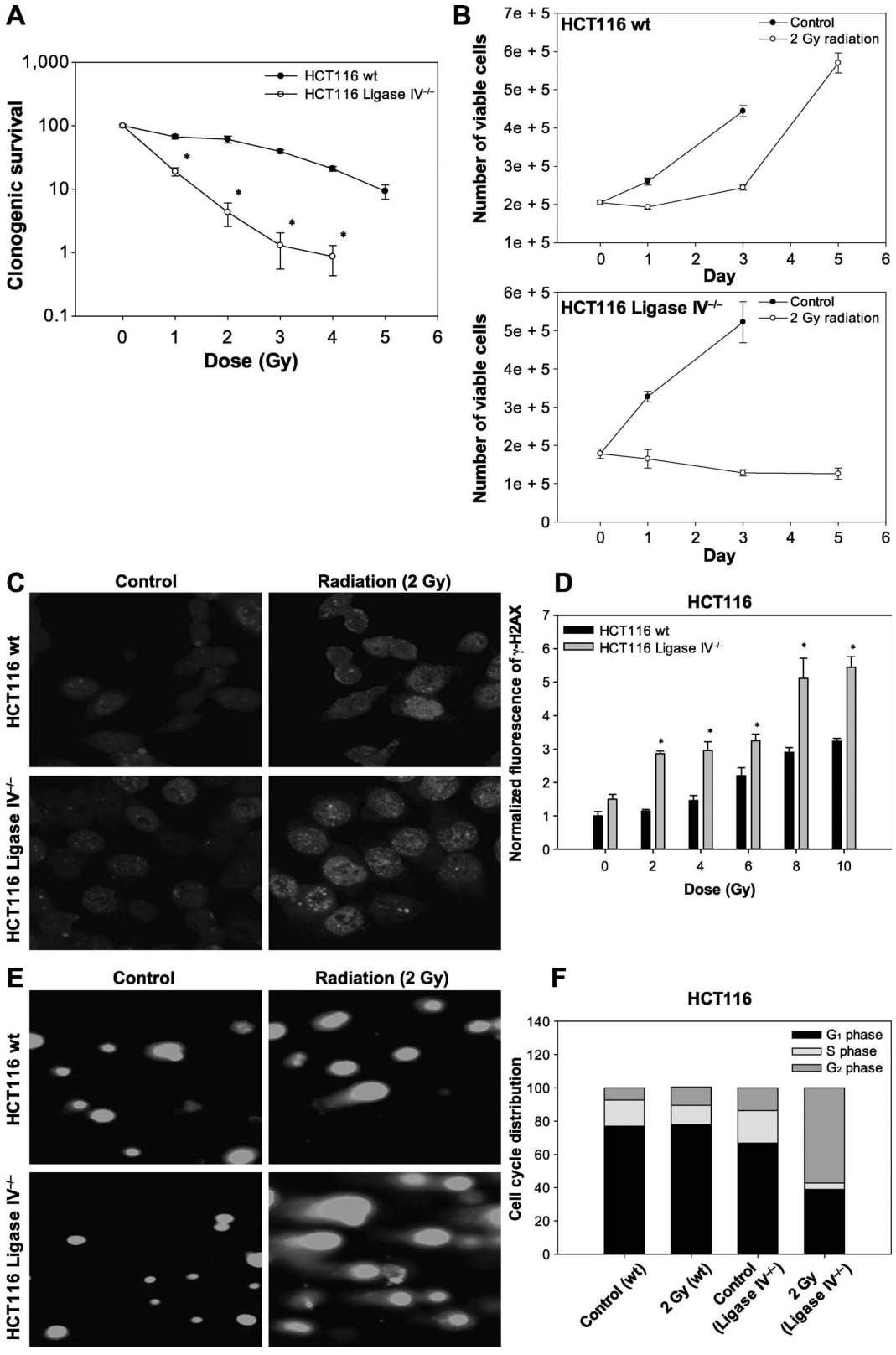

FIG. 1. Radiation responses of DNA repair-proficient and ligase IV-deficient HCT116 cell lines. Panel A: Radiosensitivity of HCT116 wild-type cells and HCT116 ligase IV-deficient cells as determined by clonogenic survival (n = 3). Panel B: Effect of radiation on cell growth. After 2 Gy irradiation of cells, the number of viable cells was determined on day 0, 1, 3 and 5 (n = 3). Graphs represent pooled data from three replicate experiments. Panel C: Confocal microscope imaging of γ-H2AX foci formation at 2 Gy irradiation at 72 h after treatment. Panel D: Mean intensity of γ-H2AX determined by flow cytometry 96 h after treatment (n = 3). Panel E: Comet

survival. Figure 1A shows that HCT116 cells lacking ligase IV were significantly more sensitive to radiation than the ligase IV wild-type cells, as the clonogenic survival was significantly decreased at lower doses of radiation compared to wild-type cells. This finding is consistent with previously published literature indicating that DNA repair-deficient cell lines are more sensitive to radiation than DNA repair-proficient cells (*40–42*).

Radiation sensitivity was further compared by performing temporal response studies in which the HCT116 and the HCT116 ligase IV-deficient cell lines were irradiated (2 Gy) and viable cell number was monitored over time. Figure 1B shows that growth of the irradiated HCT116 cells was inhibited only transiently followed by relatively rapid recovery of proliferative capacity, whereas in the irradiated HCT116 ligase IV-deficient cells, a sustained growth inhibition was observed (with a slight decline in viable cell number between day 3 and 5).

The difference in radiosensitivity of the two cell lines is likely to be a consequence of the extent and persistence of DNA damage (*42, 43*). Figure 1C (staining with γ-H2AX antibody) and D (H2AX phosphorylation) indicate that the number of DNA damage foci in HCT116 ligase $IV^{-/-}$ cells was increased compared to the ligase IV-proficient cells. That is, over a range of radiation doses, the extent of residual damage (i.e., γ-H2AX staining) at 96 h was significantly higher in the ligase IV-deficient HCT116 cells than in the ligase IV-proficient cells.

As additional confirmation of the increased DNA damage, results of comet assay experiments (Fig. 1E) show more extensive formation of tails after low-dose irradiation (2 Gy) in the HCT116 ligase $IV^{-/-}$ cells compared to the HCT116 wild-type cells. Furthermore, cell cycle analyses indicated that 72 h after a 2 Gy dose, nearly 45% of the HCT116 ligase $IV^{-/-}$ cells were arrested at the G_2/M phase compared to 20% of the HCT116 wild-type cells (Fig. 1F).

Induction of Autophagy and Senescence by Ionizing Radiation Exposure in Ligase IV-Proficient HCT116 and Ligase IV-Deficient HCT116 Cell Lines

We and others have reported that a primary response of tumor cells to radiation is autophagy (*44–47*). Figure 2A shows images of irradiated cells stained with acridine orange, which is indicative of autophagy, while Fig. 2B provides quantification of the extent of autophagy over a range of radiation doses. While the extent of autophagy was significantly greater in the ligase IV-deficient cells compared to parental cells at lower doses of radiation, essentially the entire cell population had entered a state of autophagy for both cell lines at the higher doses.

As senescence has been closely associated with autophagy in a number of published studies (*31, 48*), the induction of senescence by radiation exposure was also monitored. Both cell lines demonstrated physiological markers of senescence such as granulation, flattening and spreading, as well as β-galactosidase staining, a hallmark of senescence (Fig. 2C). In parallel to the findings relating to autophagy, senescence was more pronounced in the ligase IV-deficient cells compared to the ligase IV-proficient cells at the lower radiation doses, while higher doses yielded maximal senescent populations in both cell lines (Fig. 2D).

Although radiation-induced autophagy, senescence and persistent H2AX phosphorylation were greater in the HCT116 ligase IV-deficient cells than in ligase IV-proficient cells at the lower radiation doses, the fraction of cells showing autophagy and senescence, at any given level of γ-H2AX, was very similar for the two cell lines (Supplementary Fig. S1A and B; http://dx.doi.org/10.1667/RR14202.1.S1). Supplementary Fig. S1C also indicates a direct correspondence between the extent of autophagy and senescence (as a function of the radiation dose) in both HCT116 tumor cell lines. Thus, both senescence and autophagy correlate with, and are likely triggered by, persistent DSBs.

The Relationship between Autophagy and Senescence in Irradiated Cells

Our studies are indicative of a close correspondence between radiation-induced autophagy and senescence in both the ligase IV-deficient and the ligase IV-proficient cell lines (Supplementary Fig. S1C; http://dx.doi.org/10.1667/RR14202.1.S1), which is also the case for oncogene and chemotherapy-induced autophagy and senescence (*31, 48*). To more rigorously investigate the potential association between autophagy and senescence in response to radiation exposure, both cell lines were either pre-incubated with the pharmacological inhibitors of autophagy, chloroquine (5 μ*M*) and bafilomycin (5 n*M*), for 3 h prior to irradiation, or infected with lentivirus to induce a knockdown of the essential autophagy factors, ATG5 and ATG7. (Supplementary Fig. S2A). Supplementary Fig. S2B and C confirm that autophagy has been inhibited by chloroquine and bafilomycin in both cell lines based on the interference with radiation-induced degradation of p62/SQSTM1. Similarly, Supplementary Fig. S2D and E confirm that autophagy has been inhibited by the genetic silencing approaches. Figure 3 indicates that pharmacological and genetic inhibition of autophagy had no effect on the promotion of radiation-induced senescence in these cell lines, as the extent of senescence was essentially identical in the absence and presence of functional autophagy, strongly

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assay. Fluorescent microscope imaging of both cell lines 72 h after 2 Gy irradiation. Panel F: Cell cycle analysis after exposure of HCT116 wild-type and ligase $IV^{-/-}$ colon cancer cells to 2 Gy radiation at 72 h after treatment. Error bars represent standard error. In panels A and D, $^*P < 0.05$ compared to the corresponding effect at a similar dose of radiation in the HCT116 wild-type cells (n = 3).

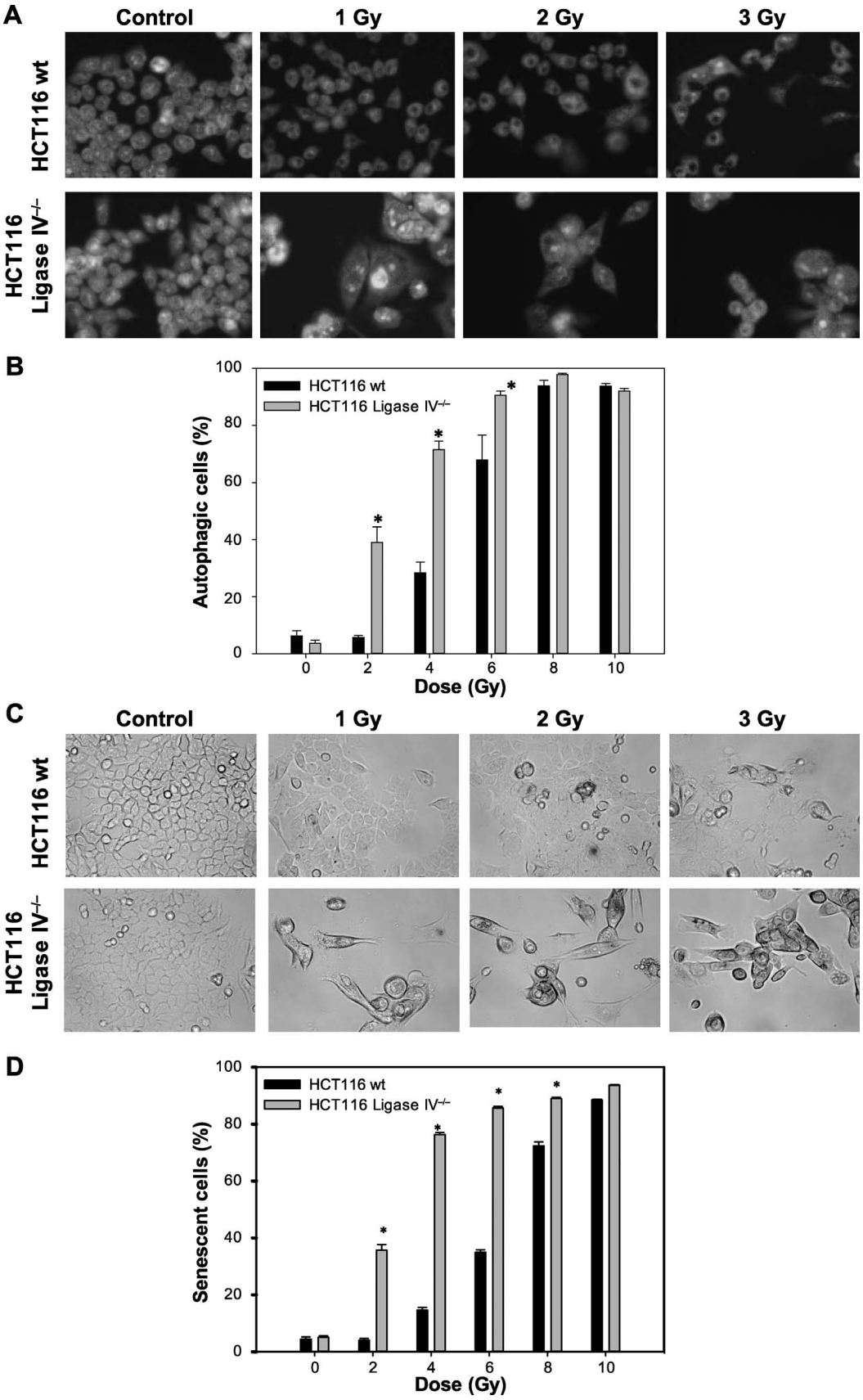

FIG. 2. Promotion of autophagy and senescence by radiation exposure in HCT116 cells. Panel A: Acridine orange staining of HCT116 wild-type and HCT116 ligase IV-deficient cells 96 h after treatment. Images shown

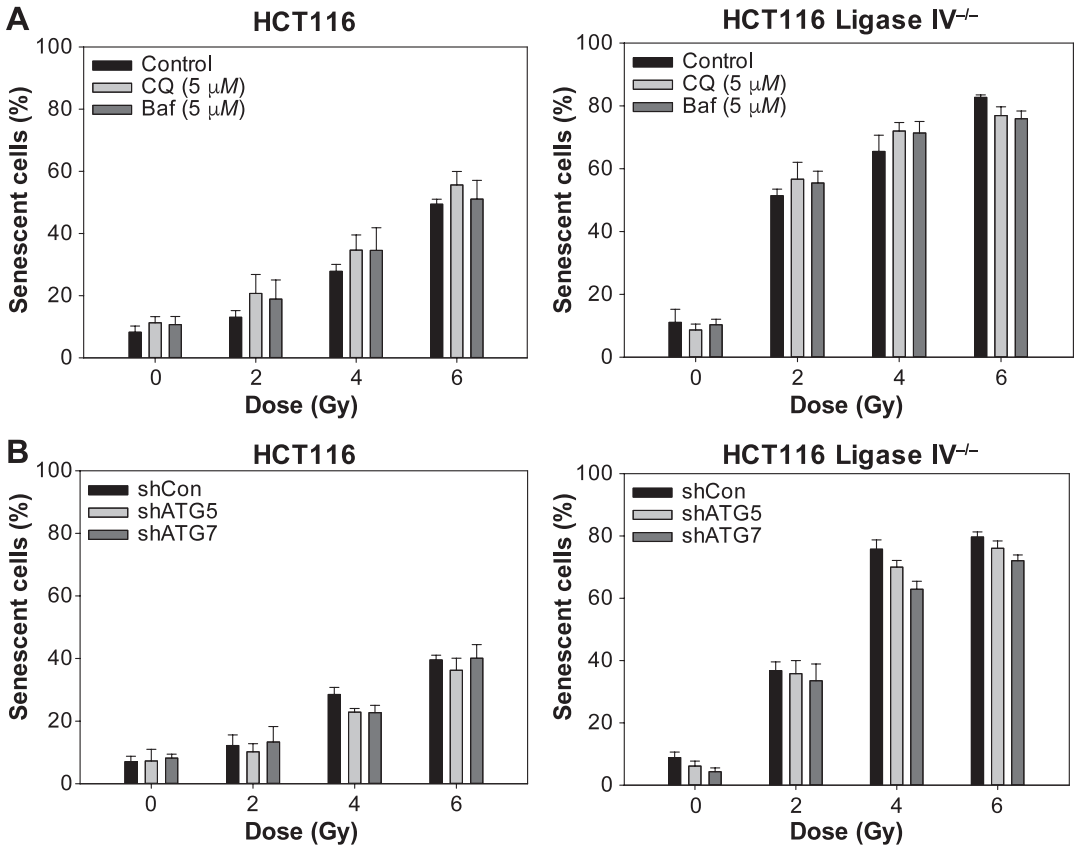

FIG. 3. Inhibition of autophagy fails to suppress radiation-induced senescence. Panel A: HCT116 cells and HCT116 ligase IV-deficient cells were pretreated with chloroquine (5 μ*M*) or bafilomycin (5 n*M*) for 3 h prior to irradiation and maintained in the presence of the autophagy inhibitors for an additional 24 h. Senescence was assessed after 96 h by flow cytometry (n = 3). Panel B: HCT116 cells and HCT116 ligase IV-deficient cells with silencing of ATG5 or ATG7 were exposed to the indicated doses of radiation and senescence was assessed after 96 h by flow cytometry (n = 3).

indicating that autophagy and senescence in response to radiation exposure are dissociable. This has, in fact, proven to be the case for both oncogene-induced senescence and senescence induced by doxorubicin (*31, 48*).

Cells Undergoing Radiation-Induced Autophagy/ Senescence Retain the Capacity for Proliferative Recovery and Are Capable of Repairing DNA DSBs

We have shown proliferative recovery after induction of senescence by radiation exposure as well as doxorubicin in breast tumor cells (*16, 31, 44, 47*). The HCT116 ligase IV-proficient and HCT116 ligase IV-deficient cells were exposed to radiation doses of 6 Gy and 3 Gy, respectively; cells were sorted based on staining with the senescence marker, C_{12}FDG, 96 h postirradiation by flow cytometry (Fig. 4A and B). Both subpopulations (i.e., positively stained and negatively stained cells) were replated at subconfluent density. Figure 4C confirms that recovery occurs after radiation-induced autophagy and senescence in both the HCT116 ligase IV-proficient and ligase IV-deficient cells. These findings are consistent with studies where proliferative recovery was observed after irradiation of MCF-7 breast tumor cells (*16, 44*).

The capacity for proliferative recovery suggests that DNA repair is likely to be functional in the autophagy/senescent cells. To address this possibility, HCT116 cells were exposed to a dose of radiation (8 Gy) that induces ~75% of both autophagy and senescence; the cells were then allowed to undergo repair for 4 days, followed by re-irradiation with 4 Gy. Repair intensity was measured after 30 min, 3 h, 24 h and 4 days based on γ-H2AX intensity determined by flow cytometry. Four days after the first irradiation (8 Gy), the level of γ-H2AX remained high. At the second irradiation (4 Gy), the intensity of γ-H2AX was

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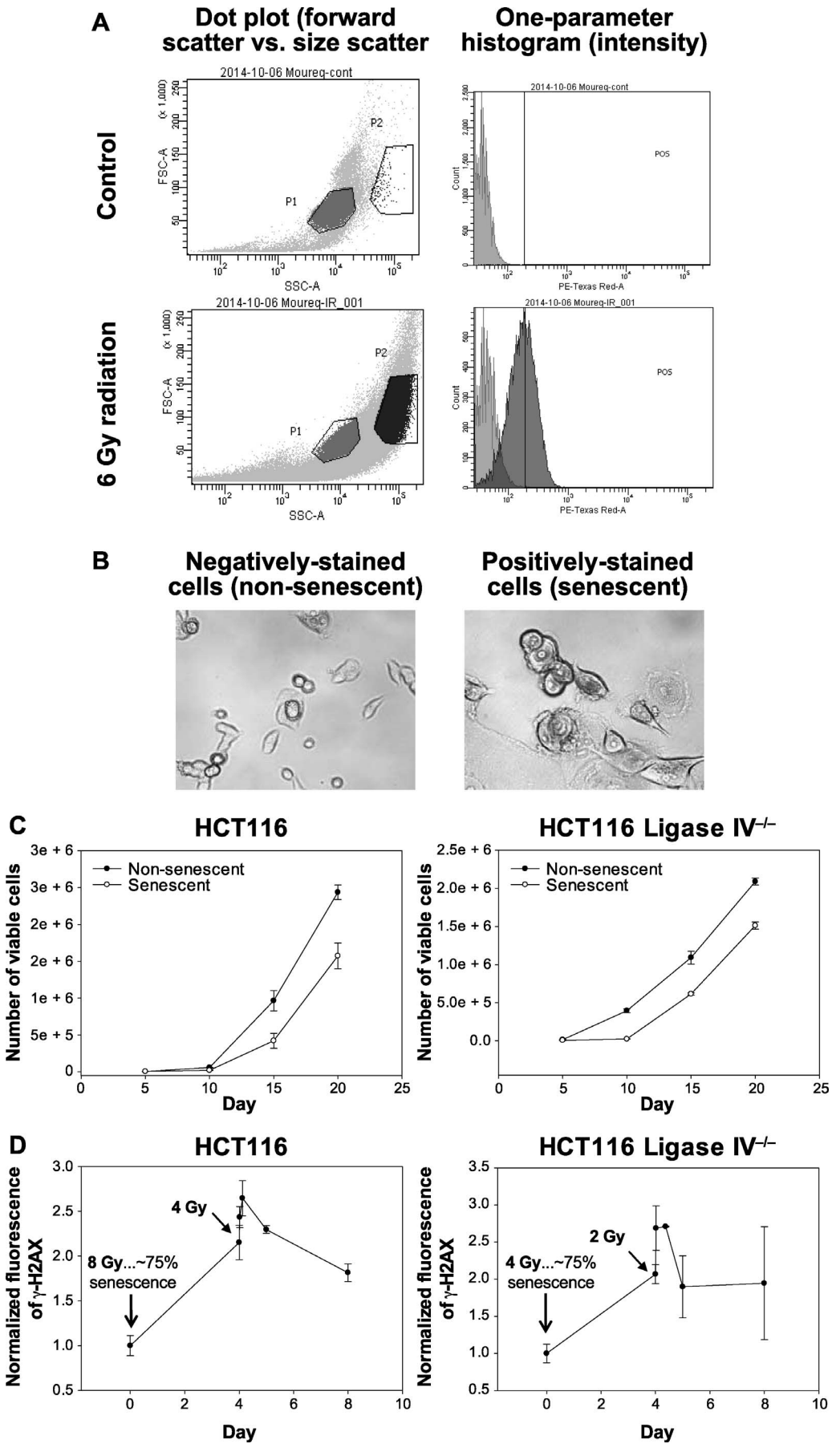
are representative of three replicate studies. Panel B: Quantification of autophagy by acridine orange flow cytometry 96 h after treatment. Error bars represent standard error. $^*P < 0.05$ compared to the corresponding radiation dose in HCT116 wild-type cells (n = 3). Panel C: Promotion of senescence based on β-galactosidase staining. Panel D: Quantification of β-galactosidase by flow cytometry at 96 h (n = 3). Error bars represent standard error. In panels B and D, $^*P < 0.05$ compared to the corresponding effect at a similar radiation dose in the HCT116 wild-type cells (n = 3).


A
Dot plot (forward scatter vs. size scatter
One-parameter histogram (intensity)
Control
6 Gy radiation
2014-10-06 Moureq-cont
2014-10-06 Moureq-IR_001
P1
P2
POS
FSC-A
SSC-A
PE-Texas Red-A
Count
B
Negatively-stained cells (non-senescent)
Positively-stained cells (senescent)
C
HCT116
HCT116 Ligase IV-/-
Non-senescent
Senescent
Number of viable cells
Day
D
HCT116
HCT116 Ligase IV-/-
Normalized fluorescence of γ-H2AX
4 Gy
8 Gy...~75% senescence
2 Gy
4 Gy...~75% senescence

further elevated for 3 h. However, the intensity of γ-H2AX was reduced by 24 and 96 h after the second dose, suggesting that, despite the persistence of initial DNA damage, these cells were still generally proficient in DNA repair capacity (Fig. 4D, left-side panel).

Similarly, HCT116 ligase IV$^{-/-}$ cells were initially irradiated with 4 Gy, a dose that induces ~75% of senescence and autophagy, followed four days later by 2 Gy irradiation. Figure 4D, right-side panel, indicates that even these ostensibly repair-incompetent cells show the capacity to repair the newly induced DNA damage.

Radiosensitization by PARP Inhibitors Correlates with Increased Autophagy and Senescence, but Not Apoptosis

There has been a great deal of interest in utilizing DNA repair inhibitors in combination with chemotherapeutic drugs and radiation to enhance the efficacy of cancer therapy. In the context of this work, it has been reported that radiosensitization by PARP inhibitors is accompanied by increased senescence (*29, 30, 49*). Given the evidence for correspondence between autophagy and senescence in the current work, we proceeded to investigate whether sensitization by PARP inhibitors could be mediated through the promotion of autophagy as well as senescence. Two different PARP inhibitors, AZD-2281 (olaparib) and MK-4827 (niraparib), were utilized to investigate whether the PARP inhibitors could sensitize both ligase IV-deficient cells and ligase IV-proficient HCT116 cells to radiation. Figure 5A shows that PARP inhibitors conferred profound radiation sensitization in the ligase IV-proficient HCT116 cells. However, while ligase IV-deficient cells were also sensitized, the degree of sensitization was clearly less than in the ligase IV-proficient cells. Temporal response data (Fig. 5B) also showed a more pronounced radiosensitization in the ligase IV-proficient cells than in the ligase IV-deficient cell lines when equitoxic doses of radiation were used. As expected, this sensitization was associated with an increase in DNA damage based on the intensity of γ-H2AX formation (Fig. 5C) and the increased DNA content in the comet tails by the comet assay (Supplementary Fig. S3A and B; http://dx.doi.org/10.1667/RR14202.1.S1).

Sensitization to radiation by the PARP inhibitors is also associated with an increase in senescence. Quantification of the intensity of β-galactosidase staining by flow cytometry indicated that between 55–60% of the ligase IV-proficient HCT116 cells had entered a state of senescence when the PARP inhibitors were used in combination with radiation, whereas radiation treatment alone induced ~20% senescence (Fig. 5D, left-side panel and Supplementary Fig. S3C). Similarly, the HCT116 ligase IV-deficient cells showed an increase in the senescent population from less than 20% to between 45–55% when the PARP inhibitor was administered along with radiation treatment (Fig. 5D, right-side panel, and Supplementary Fig. S3D). Consistent with the increased senescence, cell cycle analysis results demonstrated that ~45% of the population in both cell lines underwent growth arrest at the G_2/M phase when cells were treated with the combination compared to ~20% when cells were exposed to radiation alone (Supplementary Fig. S3E).

The combined treatment of AZD-2281 or MK-4827 with radiation also resulted in increased autophagy. Quantification of the intensity of autophagy by flow cytometry showed an increase in the number of autophagic cells to 70–80% of the population for the combination treatment in the ligase IV-proficient cells, whereas radiation alone promoted approximately 30% autophagy (Fig. 5E, left-side panel, and Supplementary Fig. S4A and B; http://dx.doi.org/10.1667/RR14202.1.S1). Similarly, the HCT116 ligase IV-deficient cells showed an increase in the autophagic population to 75% when the PARP inhibitor was administered along with radiation compared to 35% when exposed to radiation alone (Fig. 5E, right-side panel and Supplementary Fig. S4C).

Overall, the PARP inhibitors appear to produce comparable enhancement of H2AX phosphorylation, autophagy and senescence in wild-type and ligase IV-deficient cells, but less radiosensitization of ligase IV-deficient cells, particularly as measured by clonogenic survival.

Although co-treatment with PARP inhibitors enhanced the radiosensitivity of ligase IV proficient HCT116 cells and HCT116 ligase IV-deficient cells, it was critical to determine whether the cells would retain their proliferative recovery after the combination treatment by monitoring cell viability over an extended period after treatment. Figure 6 demonstrates that both cell lines recovered proliferative capacity on day 10, 15 and 20 after treatment when radiation was combined with the PARP inhibitors.

Lack of Involvement of Apoptosis in Sensitization by PARP Inhibitors in HCT116 Cells

Use of PARP inhibitors has generally been shown to radiosensitize cells through the induction of senescence, but

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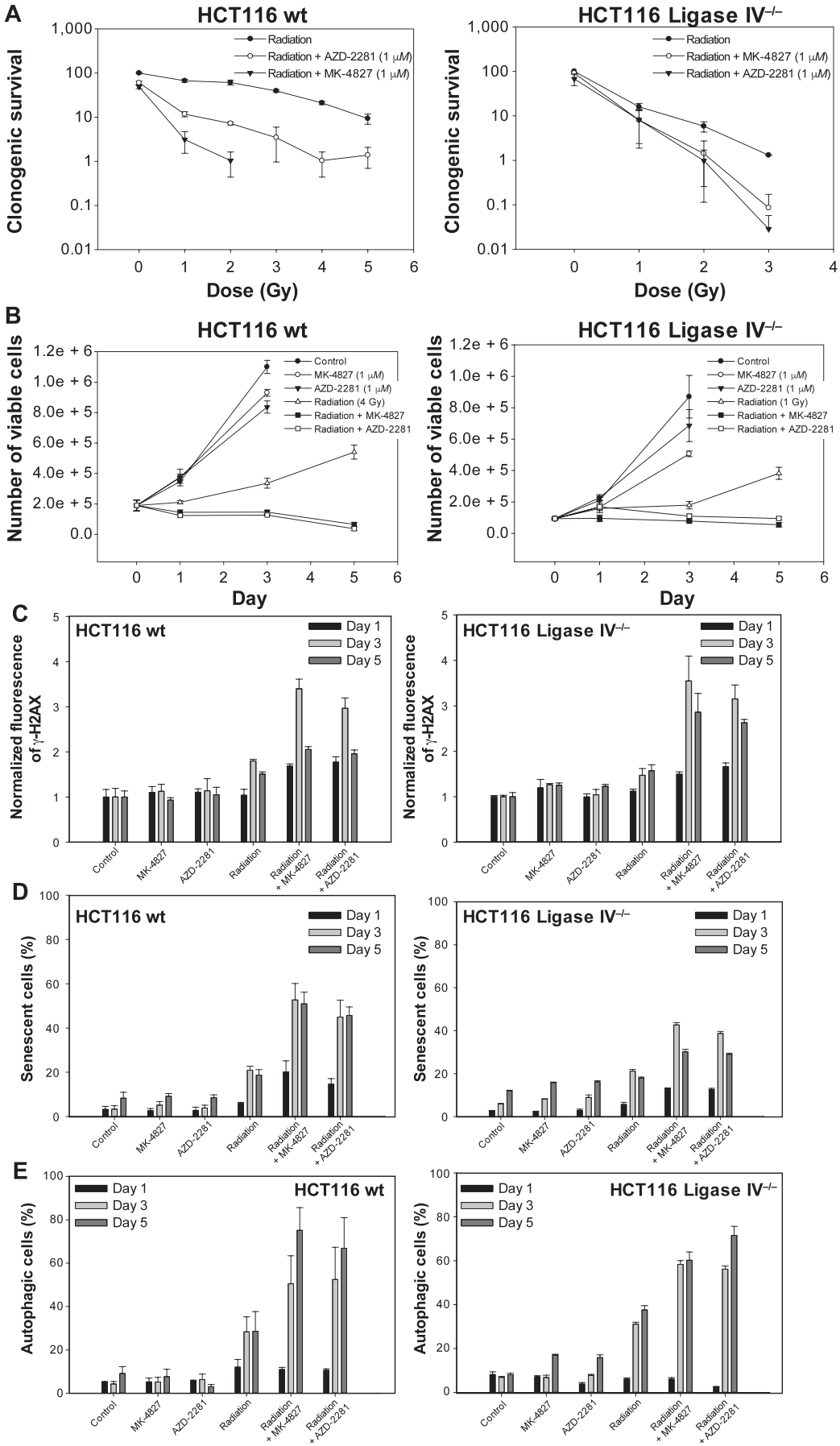
FIG. 4. DNA repair capacity in senescent cells. Panel A: HCT116 cells were stained with β-galactosidase substrate (C_{12}FDG) 96 h after treatment, and subjected to sorting by flow cytometry at excitation/emission wavelengths of 490/514 nm. Left-side panels show gating of cells based on forward scatter vs. side scatter; right-side panels show gating applied to data from 488-610/20 channel to detect β-Gal fluorescence. Panel B: Both subpopulations were stained with β-galactosidase to ensure that cells were successfully sorted according to size and fluorescence. Panel C: Senescent and nonsenescent subpopulations were replated separately in 6-well plates, and viable cell number was monitored at the indicated time points by trypan blue exclusion. Panel D: HCT116 cells and HCT116 ligase IV$^{-/-}$ cells were exposed to 8 Gy and 4 Gy doses of radiation followed by a 96 h interval for DNA repair and subsequent re-exposure to 4 Gy and 2 Gy doses of radiation, respectively. Intensity of γ-H2AX fluorescence was measured by flow cytometry at the indicated time points (n = 3).


A
HCT116 wt
Clonogenic survival
Dose (Gy)
Radiation
Radiation + AZD-2281 (1 μM)
Radiation + MK-4827 (1 μM)
HCT116 Ligase IV−/−
Radiation + MK-4827 (1 μM)
Radiation + AZD-2281 (1 μM)
B
HCT116 wt
Number of viable cells
Day
Control
MK-4827 (1 μM)
AZD-2281 (1 μM)
Radiation (4 Gy)
Radiation + MK-4827
Radiation + AZD-2281
HCT116 Ligase IV−/−
Radiation (1 Gy)
C
HCT116 wt
Normalized fluorescence of γ-H2AX
Day 1
Day 3
Day 5
HCT116 Ligase IV−/−
Control
MK-4827
AZD-2281
Radiation
Radiation + MK-4827
Radiation + AZD-2281
D
HCT116 wt
Senescent cells (%)
HCT116 Ligase IV−/−
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Autophagic cells (%)
HCT116 Ligase IV−/−

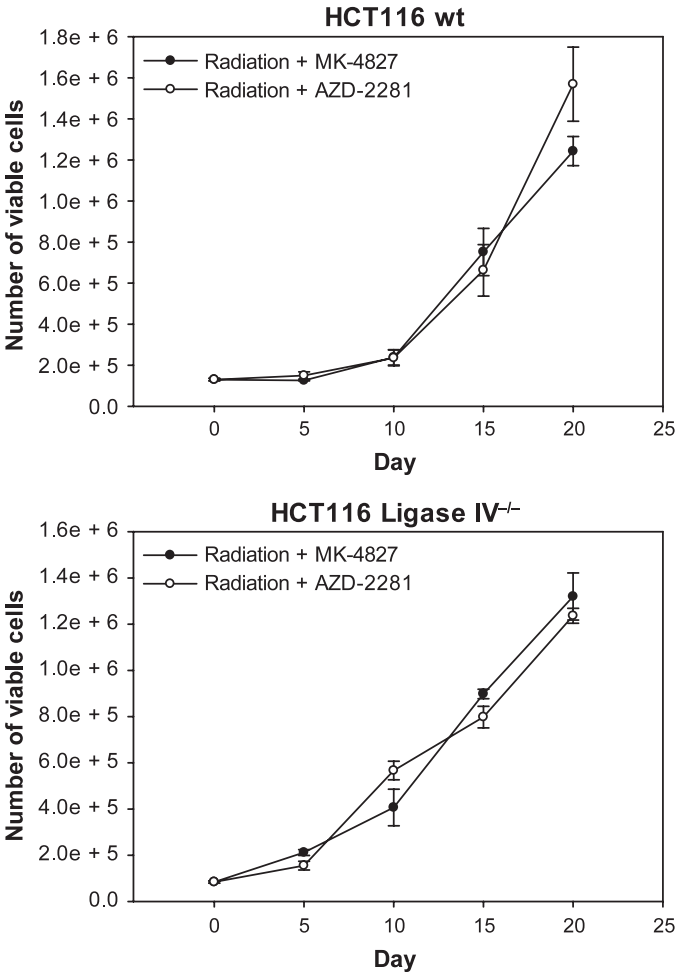

FIG. 6. Proliferative recovery after irradiation and PARP inhibition. HCT116 wild-type and HCT116 ligase IV-deficient cells were incubated with AZD-2281 (1 μ*M*) or MK-4827 (1 μ*M*) for 3 h before 4 Gy and 1 Gy doses of radiation, respectively, and maintained in the presence of the inhibitors for an additional 24 h. Viable cell number was monitored over a period of 20 days.

not apoptosis (*30, 49*). To rule out the potential involvement of apoptosis in radiosensitization, apoptotic cell death was monitored by Annexin V staining. Supplementary Fig. S5A (http://dx.doi.org/10.1667/RR14202.1.S1) indicates that apoptosis is unlikely to be involved in radiosensitization of both cell lines by PARP inhibition as apoptosis was minimal and not increased by the PARP inhibitors. The minimal involvement of apoptosis in radiosensitization by PARP inhibition was confirmed by assessment of apoptosis 72 h after treatment using the TUNEL assay (Supplementary Fig. S5B and C). To further confirm these results, irradiated HCT116 cells were treated with the pan-caspase inhibitor Z-VAD-FMK (10 μ*M*) and viable cell numbers were monitored over five days. Supplementary Fig. S5D shows that interference with apoptosis via inhibition of caspases did not interfere with radiosensitization by the PARP inhibitors, indicating that apoptosis does not appear to be involved in mediating the observed effects. Consistent with these observations, cell cycle analysis demonstrated that administering PARP inhibitors along with radiation does not increase the sub-G_1 population (data not shown), confirming that apoptosis is not occurring in cells exposed to radiation + PARP inhibitors.

Effects of Autophagy Inhibition on Radiosensitization by PARP Inhibition

Our study clearly shows that autophagy was increased in association with senescence when PARP inhibitors were used in combination with radiation. Several published studies, including our own, have demonstrated that autophagy can act as a cytotoxic or cytostatic process through which cells die or undergo prolonged growth arrest (*44, 46, 47, 50*). To address whether inhibition of autophagy would interfere with the radiosensitization by PARP inhibitors, HCT116 cell lines where autophagy was genetically silenced or pretreated with chloroquine were exposed to radiation in the absence and presence of the PARP inhibitors. Figure 7A–F shows that genetic interference with autophagy does not rescue either of the HCT116 cell lines from radiosensitization by PARP inhibition. These findings are supported by the data shown in Supplementary Fig. S6 (http://dx.doi.org/10.1667/RR14202.1.S1) where the autophagy inhibitor, chloroquine, also failed to interfere with radiosensitization by the PARP inhibitors in HCT116 cells as well as H460 non-small cell lung cancer cells, indicating that radiosensitization does not occur via the promotion of autophagy.

DISCUSSION

DNA Damage, Autophagy and Senescence Induced by Radiation

Although radiotherapy is one of the most widely used cancer therapies, its effectiveness may vary widely according to tumor type. For example, radiotherapy significantly reduces recurrence and improves outcomes in breast and head and neck cancer, respectively (*51, 52*), but is less effective in the treatment of glioblastoma and lung

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FIG. 5. Cell survival, DNA damage, autophagy and senescence in irradiated cells exposed to PARP inhibitors. HCT116 wild-type and HCT116 ligase IV-deficient cells were incubated with AZD-2281 (1 μ*M*) and MK-4827 (1 μ*M*) for 3 h prior to irradiation and maintained in the presence of the inhibitors for an additional 24 h. Panel A: The number of colonies was determined after 14 days (n = 3). Panel B: The number of viable cells was counted at the indicated time points (n = 5). Panel C: γ-H2AX intensity was measured at the indicated time points by flow cytometry in both cell lines (n = 3). Panel D: Quantification of senescence by flow cytometry at the indicated time points (n = 3). Panel E: Quantification of autophagy by flow cytometry at the indicated time points (n = 3).

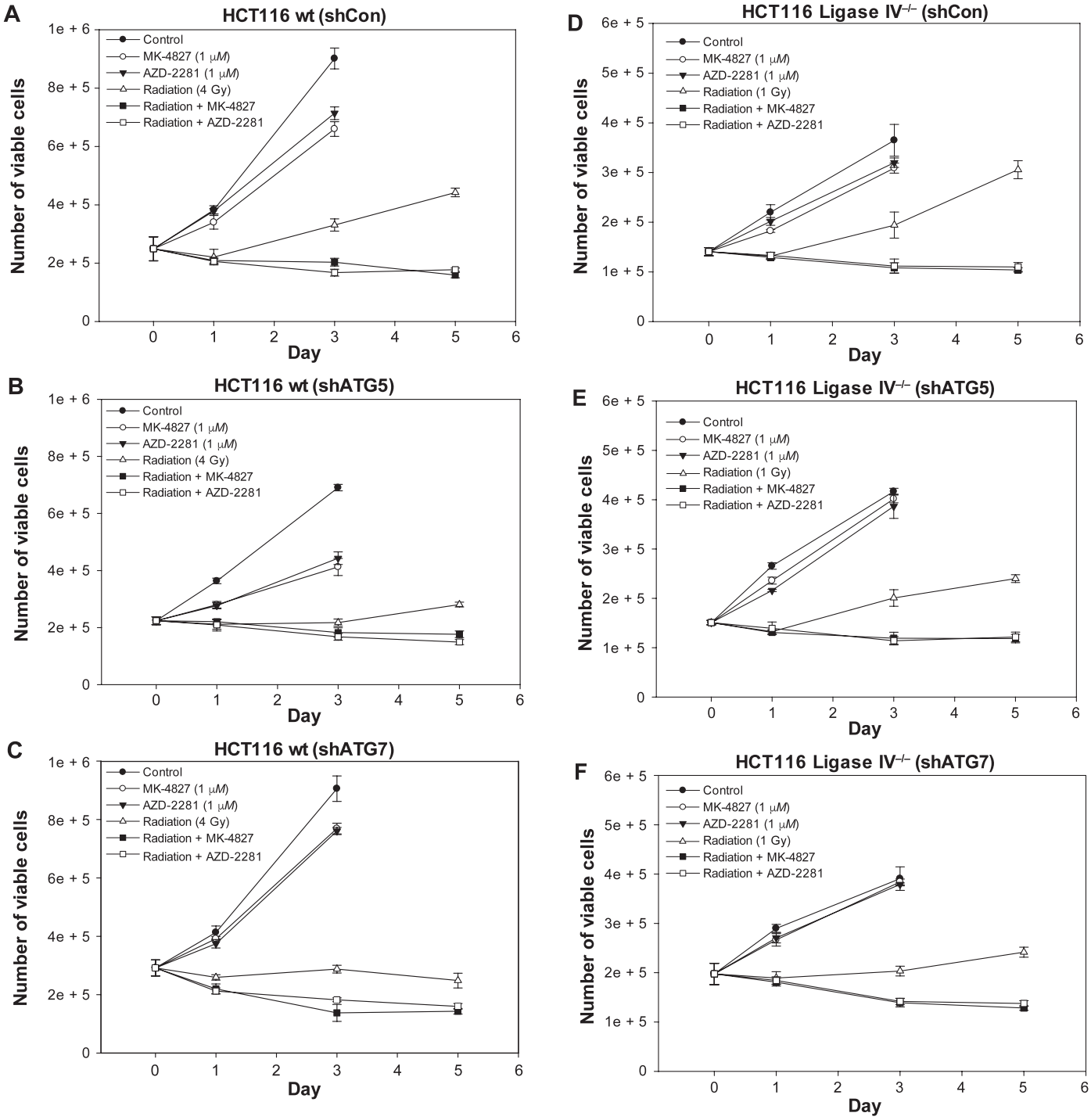

FIG. 7. Inhibition of autophagy does not alter radiosensitization by PARP inhibition. Autophagy-regulated genes were silenced in the HCT116 wild-type and HCT116 ligase IV-deficient cell lines using short hairpin RNA (shRNA) for *ATG5* and *ATG7*. Panels A–C: Autophagy-proficient HCT116 cells were irradiated with and without exposure to PARP inhibitors. Panels D–F: Autophagy-deficient HCT116 ligase IV cells were irradiated with and without exposure to PARP inhibitors.

cancer (*53–55*). Consequently, decades of preclinical efforts have been devoted to the development of strategies to sensitize malignancies to radiation therapy.

While it is generally agreed that radiation kills tumor cells by generating DSBs, these breaks as well as other DNA damage elicit a complex cascade of responses that can influence the repair and persistence of DNA damage as well as the consequences of unrepaired damage, factors that together determine whether or not an irradiated tumor cell will ultimately resume proliferation. Among these responses, radiation can induce cells to enter states of senescence as well as autophagy (*5, 56*). Because autophagy can function

as either a pro-survival or pro-death mechanism (*57, 58*), depending on the agents used and the experimental systems, it provides an especially attractive target for pharmacological manipulations that could selectively increase radiosensitivity of tumor cells but not normal cells.

The relationship between autophagy and DNA repair is unclear, but autophagy can alter the cellular response to DNA damaging agents. Disruption of autophagy by bafilomycin A1, an autophagy inhibitor, sensitized glioma cells to the alkylating agent telmozolomide by inducing apoptosis (*11*). Similarly, 6-thioguanine-induced autophagy enhanced the survival of human colorectal and endometrial cells, indicating in both studies that autophagy may play a protective role against DNA damage (*12*). Robert *et al.* recently reported on their findings that autophagy and protein acetylation are important in DNA damage repair via activation of cell cycle check points, influencing homologous recombination repair (HRR) (*13*). Another published study has shown that PARP-1 might link DNA damage to autophagy through the depletion of ATP and NAD^+, which may indicate that a cytoprotective function of autophagy was promoted to supply the cell with energy (*14*). Interference with autophagy by knocking out FIP200, an essential player in mammalian autophagy, resulted in impaired DNA repair in mouse embryonic fibroblasts treated with DNA damaging agents (*15*). On the other hand, human malignant glioma cells undergo autophagic cell death upon inhibition of the DNA-dependent protein kinase catalytic subunit (DNA–PKcs), a protein involved in non-homologous end joining (NHEJ) (*59*). In related studies, inhibition of DNA-PKcs was found to radiosensitize radioresistant prostate cancer cells by inducing autophagy (*60*). It therefore appears likely that autophagy has an important function in the enhancement of DNA repair in cells during exposure to genotoxic stress, but that the role of autophagy may differ according to the status of DNA repair.

Senescence is also induced upon exposure to a DNA-damaging agent such as radiation (*16, 17, 56*). Moreover, similar to the telomere-associated foci of replicative senescence, radiation-induced senescence is associated with persistent DNA damage foci (*61, 62*), presumably unrepaired DSBs that can remain for months and may be essential for maintaining the long-term growth arrest that characterizes the senescent cell.

In this work, we sought to identify whether autophagy and senescence play major roles in either facilitating or antagonizing DNA repair, using two isogenic cell lines, HCT116 and HCT116 ligase $IV^{-/-}$ cells. The ligase IV mutation completely inactivates repair of DSBs by classical NHEJ (*63*). Thus, as expected, at 1–2 Gy irradiation, the ligase IV-deficient cell line demonstrated lower survival than parental cells and higher levels of persistent γ-H2AX foci. At each dose of radiation this increased damage was accompanied by higher levels of both senescence and autophagy than in parental cells. Moreover, when senescence or autophagy were plotted as a function of γ-H2AX intensity at 96 h postirradiation, the plots for the two cell lines were very similar. Thus, autophagy and senescence appear to correlate with the level of persistent DSBs, suggesting that the persistent breaks and associated repair foci are primarily responsible for promoting and sustaining both senescence and autophagy. These data are consistent with studies showing that DNA damage can also induce cells to undergo a state of senescence associated with autophagy (*31*).

Relationship between Radiation-Induced Autophagy and Senescence

As we observed a direct correlation between the fractions of autophagic and of senescent cells in response to DNA damage in both cell lines, it was noteworthy to determine whether the functions of autophagy and senescence were interlinked. The relationship between autophagy and senescence is still debatable. While the induction of senescence has been reported to be, at least in part, dependent on autophagy (*31, 48*), other studies have concluded that senescence is independent of autophagy (*64, 65*). Despite the close correspondence between autophagy and senescence in parental and ligase $IV^{-/-}$ HCT116 cells, pharmacological and genetic inhibition of autophagy did not seem to affect the promotion of senescence even at high doses of radiation, indicating that promotion of senescence is independent of autophagy in this experimental model.

Effect of PARP Inhibition on Radiation Sensitivity, Autophagy and Senescence

Given the observation that irradiated cells appear to undergo proliferative recovery after a period of growth arrest, we sought to sensitize both cell lines to radiation by interfering with DNA repair to combat the recovery. PARP inhibitors are considered one of the promising radiosensitizing agents that have been tested in clinical trials (*66–71*). The poly (ADP-ribose) polymerase (PARP) enzyme is involved in repair of single-strand breaks (SSBs), and lack of this enzyme in knockout mice enhanced sensitivity to radiation and alkylating agents (*72*). PARP inhibition converts SSBs to DSBs, which in turn leads to the activation of HRR (*73*). In cells lacking the BRCA1 protein, which is a critical component of the HRR pathway, PARP inhibition can be lethal even in the absence of exogenous DNA damaging agents (*74*). Due to microsatellite instability in colorectal cancer cells, the expression of MRE11, another protein involved in the HRR pathway, is reduced (*75*). Thus, co-administration of PARP inhibitors with radiation was anticipated to lead to radiosensitization in HCT116 cells.

PARP inhibitors increased the intensity of γ-H2AX and the number of irradiated cells undergoing autophagy and senescence, but not apoptosis. These data were consistent

with our findings that both autophagy and senescence are directly correlated with induced DNA damage. In parental HCT116 cells, this increased DNA damage was associated with a dramatic reduction in clonogenic survival of irradiated cells, especially with MK-4827. Radiosensitization of the already radiosensitive ligase IV-deficient cells was less robust, despite similarly elevated levels of γ-H2AX, autophagy and senescence. This result is consistent with a model wherein radiosensitization results at least in part from inappropriate channeling of replication-associated one-sided DSBs into NHEJ; thus, when NHEJ is absent, radiosensitization is diminished. This mechanism has been invoked previously to explain the similar dependence of PARP inhibitor sensitivity on the absence of NHEJ in BRCA1-deficient cells (*76*), except that in those cells the initial SSBs would be spontaneous rather than radiation induced. In contrast to our results, a previous study reported that ligase IV-deficient mouse fibroblasts were radiosensitized by a PARP inhibitor (olaparib) at least as much as wild-type cells, and in that case radiosensitization was attributed to inhibition of a backup or ''alternative'' (Alt-NHEJ) pathway that is PARP-dependent and ligase IV-independent (*77*). Intriguingly, however, those ligase $IV^{-/-}$ cells, but not the normal cells, were also $p53^{-/-}$ due to the inviability of $p53^{+/+}$ ligase $IV^{-/-}$ mice, whereas HCT116 and its derivatives are $p53^{+/+}$. An alternative explanation for the relative lack of sensitization of the ligase $IV^{-/-}$ HCT116 cells is that these cells, after extended propagation in culture, have acquired upregulated HRR or Alt-NHEJ functions (*78*), rendering these repair systems less susceptible to PARP inhibitors.

In any case, apoptosis does not appear to be involved in the cytotoxicity of combination therapy in either cell line, indicating that the radiosensitization of these cell lines by PARP inhibitors might be mediated by promoting autophagy and senescence. Interestingly, inhibition of autophagy also did not interfere with PARP inhibitor-mediated radiosensitization, as judged by the temporal response assay. Taking into the consideration that radiation-induced senescence and radiation-induced autophagy are not linked in our system, these findings support the premise that radiosensitization is likely to be occurring via the promotion of senescence.

A recently published study showed that PARP-1 is involved in a newly identified backup pathway named PARP1-dependent end joining (PARP1-EJ) (*79*). This new finding adds another aspect of the lethality of our combination therapy in HCT116 cell lines when main repair pathways are blocked, i.e., it could account for the residual radiosensitizing effect of PARP inhibitors in the cells that lack ligase IV. However, another NHEJ-like backup repair mechanism, called the mutagenic NHEJ pathway or A-NHEJ, can be activated when PARP1-mediated and HR pathways are inactivated (*80*).

The relationship among DNA damage, autophagy and senescence is likely to be quite complex and our goals were primarily to establish whether sensitization through PARP inhibitors could occur through senescence, as postulated by the Weichselbaum group (*30, 49*), whether the senescence might be dependent on autophagy and whether autophagy might also play a role in sensitization. Our studies rule out the involvement of autophagy in the radiosensitization and furthermore dissociate radiation-induced autophagy from senescence. With regard to mechanistic questions, it has been demonstrated that DNA damage can lead to senescence and autophagy, possibly via induction of ATM. The upregulation of ATM leads to the activation of its downstream target p53, which then promotes senescence via the p21-pRb pathway (*81, 82*). Also, p53 can activate the autophagy promoter AMP-activated protein kinase during the genotoxic stress, which in turn phosphorylates tuberous sclerosis complex (TSC) proteins TSC1 and TSC2 (*83–85*). Both the TSC1 and TSC2 proteins downregulate mTOR, which eventually leads to the promotion of autophagy (*86, 87*). It is worth mentioning that HCT116 cells have a frameshift mutation in p16 (*88*), however, a number of published studies, including this current study, have indicated that senescence could be upregulated in a p16-independent manner (*5, 89, 90*). Our data suggest that a persistent DNA damage response may upregulate ATM and induce p53. It is possible that the autophagy/senescence pathways diverge at p53 wherein for senescence the pathway could involve the sequence of p53-p21-pRb, whereas in autophagy the pathway may reflect actions at the level of p53-AMPK-TSC-mTOR. We anticipate that future studies will address these questions.

Conclusions

There is no consensus as to whether radiation-induced senescence or chemotherapy is reversible (*19, 22–24, 91, 92*). We demonstrate, both in the case of radiation treatment alone and in the studies combining PARP inhibition with radiation treatment, that growth arrest is followed by proliferative recovery. In this context, the studies by Chitkova *et al.* in an apoptosis-deficient cell line (*93*) support our findings that senescence may be reversible. These observations clearly suggest that tumor cells entering a state of autophagy/senescence have the capacity to re-emerge into a proliferative state. If these findings can be extrapolated to clinical cancer, this may explain why radiation treatment is not fully effective for some types of malignancies. Furthermore, it is likely that the use of PARP inhibitors will result in only transient radiosensitization.

We conclude that the extent of radiation-induced DNA damage is accompanied by an increase of autophagy and senescence. The extent of autophagy and senescence induced at different doses of radiation was more pronounced in the ligase IV-deficient cells, which is correlated with increased levels of DNA damage. Autophagic/senescent HCT116 cells demonstrated the ability to repair the newly formed DSBs. These data may indicate

that promoting senescence alone would not have an effect on overall DNA repair system efficiency, which may explain why even the radiosensitized cells ultimately recover proliferative capacity. Current therapeutic regimens such as radiotherapy generally fail to completely eradicate the tumor cell population. This could be due, in part, to the induction of autophagy and senescence, which may be permissive for DNA repair as well as proliferative recovery that occurs even with the inclusion of PARP inhibitors, which may therefore not interfere with disease recurrence.

SUPPLEMENTARY INFORMATION

Fig. S1. Relationship between autophagy, senescence and DNA damage.

Fig. S2. Interference with autophagy in both cell lines by pharmacologic and genetic approaches

Fig. S3. Comet assay, senescence and cell cycle analysis.

Fig. S4. Autophagy in irradiated HCT116 wild-type and HCT116 ligase $IV^{-/-}$ cells exposed to PARP inhibitors.

Fig. S5. Apoptosis is not involved in the radiosensitization of HCT116 cells by PARP inhibitors

Fig. S6. Inhibition of autophagy does not alter radiosensitization by PARP inhibition.

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Perspective

Cancer Research

The Challenge of Developing Autophagy Inhibition as a Therapeutic Strategy

David A. Gewirtz

Abstract

The finding that cancer chemotherapeutic drugs and ionizing radiation often promote autophagy has provided the foundation for clinical trials combining autophagy-blocking agents with antitumor drugs and radiation. The premise driving these trials is that therapy-induced autophagy is cytoprotective; consequently, inhibition of autophagy is anticipated to sensitize malignancies to therapy. However, it is well-established that autophagy may also mediate the toxicity of antitumor drugs while evidence also exists for a nonprotective function of autophagy. Consequently, given that it cannot be predicted what form autophagy will take upon treatment with chemotherapy or radiation, the current ongoing clinical trials are likely to generate contradictory or inconsistent results, with the potential consequence that autophagy inhibition could be dismissed as therapeutic strategy based on what are essentially false-negative outcomes. Appropriate interpretation of the outcomes of these trials would require knowledge as to whether the drugs or radiation used promote the cytoprotective form of autophagy in the tumor cells as well as whether the chloroquine or hydroxychloroquine actually inhibit the autophagy. Ultimately, it will be necessary to identify those patients for whom the strategy of autophagy inhibition would be anticipated to improve the response to therapy. However, this is currently not feasible in the absence of appropriate bioassays or predictive markers for characterization of the autophagy or the effectiveness of pharmacologic approaches for autophagy inhibition in the clinic. *Cancer Res; 76(19); 5610–4. ©2016 AACR.*

Cytoprotective Autophagy in Cancer Therapy

It has long been recognized that the degradation of subcellular organelles through the process of autophagy provides energy and metabolic precursors necessary to sustain cell survival under conditions of hypoxia or nutrient deprivation (1). The concept that autophagy can also be considered a "first responder" to various other forms of stress, specifically those provoked by cancer chemotherapeutic drugs and radiation, is supported by studies in a variety of tumor cell models exposed to agents from multiple drug classes (2–6). Although many of these therapeutic modalities are clearly designed to be toxic to the tumor cell, direct survival advantages that autophagy might confer remain obscure because, with some exceptions, chemotherapeutic drugs and radiation generally are not considered to deprive the tumor cell of its metabolic and nutritional support. Nevertheless, the autophagic response to cancer therapeutics is frequently cytoprotective in function; specifically, inhibition of chemotherapy and radiation induced autophagy by either pharmacologic agents or genetic manipulation often results in a reduction in tumor cell survival if not enhanced tumor cell killing (2–13). However, as discussed in some detail below, autophagy is not always cytoprotective. Furthermore, inhibition of autophagy is likely to influence the immune response to therapy, the tumor stroma and normal tissue function.

Cytoprotective and Cytototoxic Autophagy in Cancer Therapy

Evidence that chemotherapeutic drugs and radiation promote cytoprotective autophagy is often based upon the observation that apoptosis is increased when the autophagy is inhibited through pharmacologic or genetic approaches. An increase in the extent of apoptosis supports the premise that autophagy has the capacity to interfere with induction of the apoptotic response pathway, and there is considerable evidence for crosstalk between autophagy and apoptosis (14, 15). What is frequently overlooked in many studies is that an increase in apoptosis is not necessarily or uniformly accompanied by an enhancement of drug or radiation sensitivity (16). That is, these studies may fail to demonstrate that the combined treatment with chemotherapy or radiation and pharmacologic or genetic autophagy inhibition results in a more pronounced antitumor response based on, for example, a simple assessment of viable cell number by such common methods as trypan blue exclusion, the release of lactate dehydrogenase as an indication of cell death or compromised clonogenic survival (i.e., reproductive cell death), which is generally considered the "gold standard" measurement of drug or radiation sensitivity. In fact, the autophagy induced by the therapeutic agent(s) may actually prove to be largely cytotoxic, where the autophagy itself is mediating drug or radiation killing in the tumor cell; consequently, the mode of cell death is merely being switched from autophagy to apoptosis. In this context, there is extensive evidence for the cytotoxic function of autophagy that is expressed in a host of tumor cell models and in response to a spectrum of therapeutic insults to the

Department of Pharmacology and Toxicology and Medicine and Massey Cancer Center, Virginia Commonwealth University, Richmond, Virginia.

Corresponding Author: David A. Gewirtz, Virginia Commonwealth University, 401 College Street, Richmond, VA 23298. Phone: 804-828-9523; Fax: 804-827-1134; E-mail: david.gewirtz@vcuhealth.org

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tumor cell (5). Interestingly and perhaps unexpectedly, aside from the functional distinction based on the consequences of autophagy inhibition, there is no unequivocal evidence to indicate that cytotoxic autophagy has biochemical or molecular characteristics that would distinguish it from the cytoprotective form. Although it would appear intuitive to expect that cytotoxic autophagy would reflect unrestrained and excessive degradation of cellular components, a form of self-cannibalism that would ultimately compromise cell survival, this has not actually been shown to be the case.

Clinical Trials

Given the fact that autophagy may express either cytoprotective or cytotoxic function, it is predictable that ongoing clinical trials involving the combination of the pharmacologic autophagy inhibitors chloroquine or hydroxychloroquine with chemotherapy or radiation in various forms of cancer would likely generate contradictory or at the very least equivocal results (17). That is, in those cases where the therapeutic agent promotes cytoprotective autophagy in patient tumors, autophagy inhibition should theoretically enhance tumor cell sensitivity to the radiation or the drugs inducing the autophagic response. Conversely, where autophagy is initially cytotoxic, autophagy inhibition might be anticipated to interfere with the effectiveness of therapy. Alternatively, autophagy that is initially cytotoxic might be converted to an alternative form of cell death such as apoptosis, with the consequence that drug/radiation sensitivity would essentially be unaltered. An additional caveat that may prove to significantly hamper interpretation of the outcomes of the current ongoing clinical trials is that chloroquine/hydroxychloroquine my fail to actually achieve levels in the tumor cell sufficient to inhibit autophagy. Finally, it is formally possible that sensitization to therapy that may be observed in select clinical trials could be occurring through off-target effects of the chloroquine or hydroxychloroquine and be unrelated to autophagy inhibition (18, 19). If chloroquine/hydroxychloroquine–mediated sensitization does not actually reflect a direct consequence of autophagy inhibition, then the outcome of this type of clinical trial would not be interpretable within the underlying framework of autophagy modulation for therapeutic benefit.

An additional complicating factor is that there is currently no uniformly accepted methodology for monitoring autophagy inhibition in patients' tumors. Assessment of autophagy in cell culture is facilitated by multiple assays, including but not limited to transmission electron microscopy, LC3 fluorescence microscopy, tandem mRFP/mCherry GFP fluorescence microscopy, immunohistochemistry, and SQSTM1/p62 and LC3–binding protein turnover assays (20). These approaches allow for evaluation of both autophagy induction and inhibition, the latter being critical where the cytoprotective actions of autophagy are the focus of the studies. The capacity to evaluate the onset of autophagy (and presumably its inhibition as well) *in vivo* uses a number of approaches that are similar to those for cell culture, specifically the analysis of GFP-LC3/Atg8 in transgenic mice systemically expressing GFP-LC3 or by transfection with GFP-LC3 plasmids, immunohistochemical detection of LC3 or other autophagic substrates such as SQSTM1/p62 in paraffin-embedded or fresh-frozen tissue, standard immunoblotting of these substrates, and analysis of autophagy in tissues *ex vivo* (20). Unfortunately, the clinical assessment of autophagy induction and/or inhibition does not appear to lend itself to any of the current methodologies.

There are at least three additional factors that are likely to complicate interpretation of the current clinical trials. One is the impact of autophagy induction and inhibition on the immune response, an issue that is far from being resolved. Studies from a number of investigators have suggested that factors secreted from autophagic cells, specifically damage associated molecular patterns (DAMPs) such as ATP and HMGB1 are critical for an effective immune response to eliminate the tumor (21–24); consequently, autophagy inhibition could prove to be counterproductive in a patient with a functional immune system. In contrast, it has been reported that activation of autophagy in tumor cells may promote escape from immunosurveillance and consequently autophagy inhibition could facilitate a more effective response of the immune system in conjunction with direct sensitization to therapy (25).

Another factor to consider is the potential influence of autophagy in the tumor stroma on the response to therapy (26, 27). There appears to be evidence that autophagy in tumor stroma promotes tumor growth by providing energy, suppressing the capacity of the tumor cell to undergo apoptosis as well as facilitating tumor invasiveness and metastatic potential (26, 27). If this is, in fact, the case, then interference with stromal cell autophagy should collaterally repress tumorigenicity.

Finally, autophagy has been shown to be beneficial to normal tissue function in terms of the removal of dysfunctional proteins and overall maintenance of cellular homeostasis (28). In fact, autophagic dysfunction has been associated with various neurodegenerative disorders such as Parkinson's disease, lysosomal storage disorders, and possibly diabetes (28) as well as hepatic diseases such as viral hepatitis and hepatoceullar carcinoma (29). Because it is highly unlikely that pharmacologic autophagy inhibitors would be tumor-selective in action, undesirable and possibly life threatening side effects could accompany a prolonged and sustained suppression of autophagy. Alternatively, if the autophagy inhibition is only transient, as may be the case using pharmacologic agents solely during the course of therapy, the impact on normal tissue function may not prove to be a major clinical concern.

The outcome of clinical trials performed without consideration of the possibility that autophagy induced in patient tumors may not be cytoprotective (or that the agents being tested do not, in fact, modulate autophagy in the tumor cell in the clinical setting) raises the concern that negative outcomes could undermine efforts to consider this strategy within the framework of conventional (or targeted) therapies. Given that is currently unrealistic to attempt to stratify patients according to whether autophagy induced in a clinical malignancy is cytoprotective or cytotoxic (even assuming that all of the tested therapies actually are promoting autophagy in patient malignancies), we risk the possibility of overlooking the subsets of patients whose tumors may be susceptible to sensitization via autophagy inhibition. However, not only does this field still lack biomarkers to determine, pre-therapy, which patients might be responsive to autophagy inhibition, we are also essentially blind as to how to evaluate the biochemical or molecular characteristics of tumors that are responsive, limiting the potential utility of these clinical trials to further inform the targeted development of this therapeutic strategy.

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Nonprotective Autophagy, an Additional Wrinkle in the Ether

Previous work by our laboratory as well as that from the Thorburn research group has identified an additional functional form of autophagy, which we have termed "nonprotective" (4, 18, 30, 31). Unlike cytoprotective autophagy, where inhibition results in an enhanced response to the therapeutic agent, or cytotoxic autophagy where inhibition is anticipated to lessen the impact of the therapeutic agent, inhibition of nonprotective autophagy fails to influence drug or radiation sensitivity. This was found to be the case for cisplatin and radiation in 4T1 and Hs578t breast tumor cells, doxorubicin in MCF-7 breast tumor cells and radiation in a variety of tumor cells lacking functional p53 (18, 30–32).

A recent article by Eng and colleagues (19) essentially confirms these findings identifying the nonprotective function of autophagy and further builds upon this concept in experiments where the *ATG7* autophagy gene has been silenced in the A549 non–small cell lung cancer (NSCLC) cell line. In studies where ionizing radiation as well as more than 30 drugs exhibiting a variety of mechanisms were tested, it was found that (with only a few exceptions) inhibition of autophagy left the IC_{50} virtually unchanged. Although it is unclear whether all of the therapeutic modalities examined actually promoted autophagy in the A549 cells, it can be assumed that this would be the case for the vast majority of the tested drugs. Furthermore, although the influence of autophagy inhibition on drug activity was not tested in tumor bearing animals, the conclusions of this work are strengthened by the fact that *ATG7* silencing clearly eliminated survival of the A549 cells under conditions of nutrient starvation.

Although the outcome of these studies clearly support previous findings that identified the nonprotective function of autophagy (18, 30–32), studies by other laboratories, including our own, frequently identified cytoprotective autophagy in NSCLC cell lines. For instance, we reported that radiation promoted protective autophagy in H460 NSCLC cells (33). However, as we do observe that etoposide promotes the nonprotective form of autophagy in the same cell line (unpublished observations), our work tends to suggest that whether autophagy is protective or nonprotective in a particular tumor cell line may depend on the nature of the treatment modality. In other studies, involving radiation-induced autophagy, where *p53* was either induced or silenced in isogenic experimental systems, we reported that cytoprotective autophagy required the cells to express functional p53 whereas in cells that are either null or mutant in p53, radiation-induced autophagy was nonprotective (31).

In a 2011 study by Han and colleagues (7) where gefitnib and erlotinib were shown to promote autophagy in A549 and H1299 NSCLC cell lines, chloroquine as well as silencing of *ATG5* and *ATG7* enhanced sensitivity to these tyrosine kinase inhibitors. These findings were confirmed and extended in a report by Zou and colleagues (8) where sensitivity to erlotinib was increased by silencing of *ATG5* in H460 and A549 cells and by exposure to chloroquine in H460, A549, H358, and H322 cells. In the latter work, the presence of chloroquine also substantially increased apoptosis; however, the effect of *ATG5* silencing on apoptosis was minimal, involving no more than 10% of the cell population. Both Ren and colleagues (9) and Wu and colleagues (10) reported that 3-methyl adenine enhanced sensitivity to cisplatin in A549 cells, albeit in an A549 cell line that had been selected for cisplatin resistance; however, there were no confirmatory genetic silencing studies and it is recognized that 3-MA is not necessarily specific for autophagy as a cellular target. Wang and colleagues (11) showed modest sensitization to topotecan and enhancement of apoptosis in A549 cells by chloroquine and with genetic silencing of *ATG5*. Pan and colleagues (12) reported sensitization to 5-fluorouracil by 3-MA and by silencing of *ATG7* in A549 cells along with an increase in apoptotic cell death while Park and colleagues (13) reported sensitization to pemetrexed, also by 3-MA, in A549 cells. The divergence in experimental outcomes suggests that it cannot be assumed that the recent findings reported by Eng and colleagues in A549 cells (19) can be extrapolated to conclude that autophagy in non-small cell lung cancer is uniformly nonprotective. Nonetheless, taken together, these reports clearly indicate that autophagy in response to radiation or chemotherapy can be either cytoprotective or nonprotective in function.

There is a quite extensive body of literature where the impact of autophagy has been evaluated using a spectrum of chemotherapeutic drugs or radiation in various experimental tumor models, primarily cell culture and tumor xenografts. Suffice it to say, when examined carefully, the results of these studies are, at the very least, inconsistent in terms of whether autophagy induced by chemotherapy and radiation is cytoprotective and amenable to manipulation for therapeutic benefit. Although it is beyond the scope of this commentary to summarize the outcome of these studies, we have recently published a focused review of the literature relating to autophagy inhibition in NSCLC models in response to chemotherapy and radiation both in cell culture and *in vivo* (34). The reader is also directed to a recently published review relating to the capacity of autophagy inhibition to sensitize tumors to radiation (35)

Where Do We Go from Here?

It is understandable that the oncology community has hastened to initiate clinical trials of autophagy inhibition as an adjunct to standard therapies, working under the premise that autophagy could represent a global mechanism of drug and radiation resistance. However, in retrospect, given the existence of at least three functional forms of autophagy in response to chemotherapy and radiation (cytoprotective, cytotoxic and nonprotective), the issue now is clearly considerably more complex. Fundamentally, the challenge is how to determine when therapy-induced autophagy is actually cytoprotective in patients to identify those patients for whom autophagy inhibition might prove to be beneficial. The governmental website (Clinical trials.org) indicates that clinical trials involving autophagy inhibition are ongoing involving, for example, gemcitabine/Abraxane in pancreatic cancer, chemoradiation for glioblastoma, RAD001 in renal cell carcinoma, Ixabepilone in metastatic breast cancer, and FOLFOX/bevacizumab in colorectal cancer. We have recently commented on the results of published clinical trials relating to autophagy inhibition, where we noted that "it might be prudent to develop a consensus based on preclinical data as to which types of cancer and which class or classes of drugs used in standard regimens might be most appropriate for testing in the context of clinical trials of HCQ or other modulators of autophagy" (17).

The strategy of autophagy inhibition would greatly benefit from the identification of serum markers that might be indicative of cytoprotective autophagy in response to a first round of therapy. However, we are currently far removed from knowing

what form such markers might take since even established disease markers such as prostate-specific antigen for prostate cancer have somewhat controversial prognostic significance. This is not to say that this goal cannot be achieved as it is now feasible to predict which patients might be susceptible to trastuzumab based on Her/neu expression in breast cancer and to tyrosine kinase inhibitors such as Gefitinib and erlotinib in lung cancer based on EGFR status. However, even in these more mature areas of oncology, a determination of the appropriate therapy is dependent on a tumor biopsy. In the case of the different forms of autophagy, the appropriate marker or panel of markers remains to be identified.

Given the limitations of our current knowledge in this field, the most effective strategy might be to obtain a biopsy with a sufficient number of cells that could be grown in culture and tested for responsiveness to a particular drug or panel of drugs in the absence and presence of an autophagy inhibitor. Not only would this provide information as to the nature of the autophagy induced by the therapy, but presumably this approach would also indicate whether the autophagy inhibitor is effective in sensitizing the tumors to the therapy. Furthermore, an indication as to the extent of sensitization that might be anticipated is likely to provide guidance for deciding whether there is likely to be therapeutic value in proceeding with this approach.

It is, of course, possible that some classes of malignancies are generally susceptible to autophagy inhibition as a sensitization strategy, although preclinical data in cell culture suggest this is unlikely to be the case. How this would be determined for patients is unclear. The current strategy adopted by the NIH as well as many laboratories involves the testing of therapeutic strategies using patient-derived tumors grown as xenografts, which is thought to be more predictive of clinical outcomes than most previous models (36, 37). In this context, in a study by Zinn and colleagues (38) chloroquine was shown to sensitize a small-cell lung cancer xenograft model to the Bcl-2 inhibitor ABT-737, but not when using patient derived tumor xenografts.

One strategy might be to screen large samples of patient derived tumors from a particular malignancy with a panel of drugs that are generally used for that disease to determine whether autophagy is induced and/or whether an autophagy inhibitor enhances drug or radiation sensitivity. However, even when these tumors are studied in tumor bearing animals, the potential involvement of the immune system will not be factored into the outcome since xenografts are obligatorily grown in immune suppressed models; nevertheless, as indicated above, it is possible if not likely that the immune response will be the ultimate determinant as to whether the strategy of autophagy inhibition will be successful in improving patient response to chemotherapy or radiation. It further remains a matter of conjecture as to what the percentage of patient-derived tumors would have to show positive responses and the extent of these responses that might be necessary for autophagy inhibition to be considered as having clinical applicability.

Given the relative paucity of preclinical information as to what might constitute cytoprotective autophagy in a patient's malignancy (vs. the cytotoxic and nonprotective forms), even if subpopulations of patients have disease that responds positively to autophagy inhibition in combination with conventional therapies, we are currently unable to identify what genetic background of the tumor or biochemical/molecular characteristics could be used as predictive factors for further application of this therapeutic strategy. This type of information (along with an assessment of the immune response and attention to the impact of autophagy on normal tissue), will ultimately be critical to providing the framework necessary to interpret whether the ongoing clinical trials will ultimately support the incorporation of pharmacologic autophagy inhibitors as a component of cancer therapy.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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The Challenge of Developing Autophagy Inhibition as a Therapeutic Strategy

David A. Gewirtz

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Effects of paclitaxel on the development of neuropathy and affective behaviors in the mouse

Wisam Toma [a, *, 1], S. Lauren Kyte [a, **, 1], Deniz Bagdas [b], Yasmin Alkhlaif [a], Shakir D. Alsharari [c], Aron H. Lichtman [a], Zhi-Jian Chen [d], Egidio Del Fabbro [e], John W. Bigbee [f], David A. Gewirtz [a], M. Imad Damaj [a]

[a] Virginia Commonwealth University, School of Medicine, Department of Pharmacology and Toxicology, Richmond, VA, 23298, USA
[b] Uludag University, Experimental Animals Breeding and Research Center, Faculty of Medicine, Bursa, Turkey
[c] King Saud University, College of Pharmacy, Department of Pharmacology and Toxicology, Riyadh, Saudi Arabia
[d] Virginia Commonwealth University, School of Medicine, Department of Neurology, Richmond, VA, 23298, USA
[e] Virginia Commonwealth University, School of Medicine, Department of Internal Medicine, Richmond, VA, 23298, USA
[f] Virginia Commonwealth University, School of Medicine, Department of Anatomy and Neurobiology, Richmond, VA, 23298, USA

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ABSTRACT

Paclitaxel, one of the most commonly used cancer chemotherapeutic drugs, effectively extends the progression-free survival of breast, lung, and ovarian cancer patients. However, paclitaxel and other chemotherapy drugs elicit peripheral nerve fiber dysfunction or degeneration that leads to peripheral neuropathy in a large proportion of cancer patients. Patients receiving chemotherapy also often experience changes in mood, including anxiety and depression. These somatic and affective disorders represent major dose-limiting side effects of chemotherapy. Consequently, the present study was designed to develop a preclinical model of paclitaxel-induced negative affective symptoms in order to identify treatment strategies and their underlying mechanisms of action. Intraperitoneal injections of paclitaxel (8 mg/kg) resulted in the development and maintenance of mechanical and cold allodynia. Carboplatin, another cancer chemotherapeutic drug that is often used in combination with paclitaxel, sensitized mice to the nociceptive effects of paclitaxel. Paclitaxel also induced anxiety-like behavior, as assessed in the novelty suppressed feeding and light/dark box tests. In addition, paclitaxel-treated mice displayed depression-like behavior during the forced swim test and an anhedonia-like state in the sucrose preference test. In summary, paclitaxel produced altered behaviors in assays modeling affective states in C57BL/6J male mice, while increases in nociceptive responses were longer in duration. The characterization of this preclinical model of chemotherapy-induced allodynia and affective symptoms, possibly related to neuropathic pain, provides the basis for determining the mechanism(s) underlying severe side effects elicited by paclitaxel, as well as for predicting the efficacy of potential therapeutic interventions.

1. Introduction

Various neoplastic diseases, such as breast, lung, and ovarian cancer, are commonly treated with paclitaxel, a chemotherapeutic drug in the taxane class. The anti-tumor effect of paclitaxel is mediated through its binding to microtubules of the cytoskeleton and enhancement of tubulin polymerization, thereby resulting in cell cycle arrest, and ultimately apoptotic cell death (Jordan and Wilson, 2004). Although paclitaxel effectively increases both progression-free survival and overall survival in cancer patients, it also produces painful sensory and emotional deficits (Dranitsaris et al., 2015; Seretny et al., 2014). Specifically, paclitaxel causes chemotherapy-induced peripheral neuropathy (CIPN), a result of peripheral nerve fiber dysfunction or degeneration, acutely in 59–78% of cancer patients and chronically in 30% of cancer patients

* Corresponding author. Virginia Commonwealth University, Hermes A. Kontos Medical Sciences Building, 1217 E Marshall St, Rm 334, Richmond, VA, 23298, USA.
** Corresponding author. Virginia Commonwealth University, Hermes A. Kontos Medical Sciences Building, 1217 E Marshall St, Rm 334, Richmond, VA, 23298, USA.
E-mail addresses: tomawb@vcu.edu (W. Toma), kytesl@vcu.edu (S.L. Kyte).
[1] These authors contributed equally to this work.

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(Beijers et al., 2012). CIPN is characterized by sensory symptoms such as numbness, tingling, cold and mechanical allodynia, as well as an overall decrease in quality of life. In addition, cancer patients receiving chemotherapy experience behavioral symptoms including fatigue, anxiety, and depression. For example, approximately 58% of cancer patients suffer from depression, while anxiety is prevalent in approximately 11.5% of the cancer patient population (Massie, 2004; Mehnert et al., 2014). Importantly, patients with comorbidities of depression and anxiety suffer from increased severity of symptoms and experience delayed recovery, which may interfere with positive outcomes (Massie, 2004). In comparison, 34% and 25% of the general population of patients experiencing neuropathic pain report respective feelings of depression and anxiety (Gustorff et al., 2008).

It is clear that there is a critical need to determine the mechanisms underlying these behavioral symptoms elicited by cancer chemotherapy drugs, as well as to identify new targets to prevent or treat these side effects. A necessary requisite to accomplish these aims is to establish relevant preclinical models of chemotherapy-induced side effects. However, to our knowledge there are presently no published preclinical studies that have characterized paclitaxel-induced affective-like behaviors. Thus, the objectives of the current study were to develop a mouse model of paclitaxel-induced side effects. Multiple assessments of nociceptive and affective-related behaviors were performed in mice treated with one cycle of paclitaxel (i.p., every other day for a total of four injections). After determining the dose-response curve and time-course of paclitaxel-induced mechanical and cold allodynia following systemic administration in mice, the impact of paclitaxel was assessed on multiple affective behavioral phenotypes in individual cohorts of mice, such as nest building, anxiety- (light/dark box test, novelty suppressed feeding), depression- (forced swim test), and anhedonia- (sucrose preference test) related behaviors. In addition, studies investigated the nociceptive effect of carboplatin treatment alone and in combination with paclitaxel due to the use of the carboplatin-paclitaxel combination in the clinic.

2. Methods

2.1. Animals

Adult male C57BL/6J mice (8 weeks at beginning of experiments, 20–30 g) were purchased from The Jackson Laboratory (Bar Harbor, ME). A total of 197 mice were used, with 84 used to assess nociceptive effects and 113 used to assess affective-like behaviors. Mice were housed in an AAALAC-accredited facility in groups of four, then individually housed for the duration of the nesting, novelty suppressed feeding (NSF), and sucrose preference assays in order to accurately assess the ability of each individual mouse to nest, and to measure the food or sucrose consumed by each individual mouse. Mice were group-housed for all other behavioral assays. Food and water were available *ad libitum*, except when under the food restrictions of the NSF assay. The mice in each cage were randomly allocated to different treatment groups. All behavioral testing on animals was performed in a blinded manner; behavioral assays were conducted by an experimenter blinded to the treatment groups. Experiments were performed during the light cycle (7:00 a.m. to 7:00 p.m.) and were approved by the Institutional Animal Care and Use Committee of Virginia Commonwealth University and followed the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals. Animals were euthanized via CO_2 asphyxiation, followed by cervical dislocation. Any subjects that showed behavioral disturbances unrelated to chemotherapy-induced pain were excluded from further behavioral testing. Animal studies are reported in compliance with the ARRIVE guidelines (Kilkenny et al., 2010).

2.2. Drugs

Paclitaxel and carboplatin were purchased from Tocris (Bristol, United Kingdom). Paclitaxel was dissolved in a mixture of 1:1:18 [1 vol ethanol/1 vol Emulphor-620 (Rhone-Poulenc, Inc., Princeton, NJ)/18 vol distilled water]. Carboplatin was dissolved in 0.9% saline. All injections were administered intraperitoneally (i.p.) in a volume of 1 ml/100 g body weight.

2.3. Induction of CIPN model

In the clinic, low-dose paclitaxel therapy consists of administering 80 mg/m^2 intravenously once every week; the duration of treatment is dependent upon disease progression and limiting toxicity (Seidman et al., 2008). To mimic this low-dose regimen, our studies involved i.p. injections of 2, 4, or 8 mg/kg paclitaxel every other day for a total of four injections (1 cycle), resulting in a cumulative human equivalent dose of 28.4–113.5 mg/m^2 (Reagan-Shaw et al., 2007). A low-dose regimen (8 mg/kg, 1 cycle) results in long-term mechanical allodynia, which better represents the clinical manifestation of peripheral neuropathy, and allows for affective-related behavioral measures to not be obscured by severe motor deficits and weight loss. When referring to the time at which affective behavioral assays were conducted, "post-paclitaxel injection" refers to the time after the first of four paclitaxel injections.

2.3.1. Immunohistochemistry and quantification of intra-epidermal nerve fibers (IENFs)

The staining procedure was based on a previously described method of Bennett et al. (2011) with modifications. The glabrous skin of the hind paw was excised, placed in freshly prepared 4% paraformaldehyde in 0.1 M PBS (pH 7.4), and stored overnight at 4 °C in the same fixative. The samples were embedded in paraffin and sectioned at 25 μm. Sections were deparaffinized, washed with PBS, and incubated at room temperature for 30 min in blocking solution (5% normal goat serum and 0.3% Triton X-100 in PBS). Sections were incubated with a 1:1000 dilution of the primary antibody, PGP9.5 (Fitzgerald - cat# 70R-30722, MA, USA) overnight at 4 °C in a humidity chamber. Following PBS washes, sections were incubated for 90 min at room temperature with a 1:250 dilution of goat anti-rabbit IgG (H+L) secondary antibody conjugated with Alexa Fluor® 594 (Life Technologies - cat# A11037, OR, USA). Sections were mounted in Vectashield (Vector Laboratories, Burlingame, CA, USA) and examined using a Zeiss Axio Imager A1 – Fluorescence microscope (Carl Zeiss, AG, Germany). Sections were examined in a blinded fashion under 63× magnification. The IENFs in each section were counted in a blinded fashion and the density of fibers is expressed as fibers/mm. An individual cohort consisting of 6 mice per group was used.

2.3.2. Cycles of paclitaxel

To investigate the impact of paclitaxel treatment on peripheral sensitization following repeated cycles, we used the lowest paclitaxel dose in this study for a total of two cycles. Mice were injected with vehicle or paclitaxel (2 mg/kg) for each cycle. Mechanical thresholds were evaluated between the days of injection and subsequently once per week. The second cycle of treatment began one week after the first cycle. An individual cohort consisting of 6 mice per group was used.

2.3.3. Carboplatin-paclitaxel treatment

In this study, we first investigated if carboplatin, which is often used in combination with paclitaxel for chemotherapeutic

intervention, would induce mechanical allodynia in mice on its own after systemic administration. To explore the effect of carboplatin on changes in nociceptive behavior, mice were injected with carboplatin (0, 5, or 20 mg/kg) for 1 cycle and tested for 7 days. In a separate experiment, we studied the impact the carboplatin treatment on paclitaxel-induced allodynia using the sequence of carboplatin-paclitaxel administration. Mice were first injected with carboplatin (5 mg/kg, 1 cycle), then another cycle of injections was administered with a low dose of paclitaxel (1 mg/kg). The second cycle of treatment (paclitaxel, 1 mg/kg) began one week following the first cycle (carboplatin, 5 mg/kg). Mechanical thresholds were evaluated between the days of injection. An individual cohort consisting of 6 mice per group was used.

2.4. Assessment of nociceptive behavior

An individual cohort consisting of 6 mice per group was used for the assessment of mechanical and cold allodynia; the mice had a resting period of 24 h between assays. An additional cohort consisting of 6 mice per group was used for the locomotor activity test to assess potential paclitaxel-induced motor deficits.

2.4.1. Mechanical allodynia evaluation (von Frey test)

Mechanical allodynia thresholds were determined using von Frey filaments according to the method suggested by Chaplan et al., (1994) and as described in our previous report (Bagdas et al., 2015). The mechanical threshold is expressed as log10 (10 £ force in [mg]).

2.4.2. Cold allodynia evaluation (acetone test)

This test was conducted as previously described (Otrubova et al., 2013), but with slight modifications. Briefly, mice were placed in a Plexiglas cage with mesh metal flooring and allowed to acclimate for 30 min before testing. 10 μl of acetone was projected via air burst from the pipette onto the plantar surface of each hind paw. Time spent licking, lifting, and/or shaking the hind paw was recorded by a stopwatch over the course of 60 s.

2.5. Locomotor activity test

The test was performed as described previously in Bagdas et al., (2015). Briefly, mice were placed into individual Omnitech (Columbus, OH) photocell activity cages (28 × 16.5 cm) containing two banks of eight cells each. Interruptions of the photocell beams, which assess walking and rearing, were then recorded for the next 30 min. Data are expressed as the number of photocell interruptions.

2.6. Assessment of affective behaviors

2.6.1. Nesting procedure

The nesting procedure was adapted as previously described by Negus et al., (2015) with some modifications. Briefly, mice were housed individually in cages containing corn cob bedding and all previous nesting material was removed from the home cage prior to conducting the nesting assay. For each cage, one compressed cotton nestlet was weighed and cut into 6 rectangular pieces of equal size. The mice were then relocated to a quiet, dark room. After an acclimation period of approximately 30 min, the nestlet pieces were then placed on top of the wire cage lid, parallel to the wire and evenly spaced. The mice were allowed 120 min to nest, after which the weight of the nestlet pieces remaining on the cage lid and the nest quality (0–2; 0 = no nest formed, 1 = some nesting activity, 2 = established nest) was recorded. The percentage of animals that did nest, the amount of nesting material acquired (percent weight used), and the ability to participate in innate murine nesting behavior (nest quality) were determined. The nesting assay was conducted with three individual cohorts of mice: one at 1 week (n = 6 per group), one at 2 weeks (n = 6 per group), and another at both 8 and 11 weeks (n = 6 Veh, n = 7 PAC) post-paclitaxel (8 mg/kg, i.p) or vehicle injection. These specific cohorts were used for both the nesting and NSF assays, since nesting is not thought to be a stress-inducing task. The mice had a resting period of one week between assays.

2.6.2. Novelty suppressed feeding (NSF)

The NSF test measures a rodent's aversion to eating in a novel environment. It assesses stress-induced anxiety by measuring the latency of an animal to approach and eat a familiar food in an aversive environment (Bodnoff et al., 1988). Mice were housed individually in cages with wood-chip bedding and were deprived of food for 24 h. At the end of the deprivation period, the mice were relocated to a quiet, dark room. After an acclimation period of approximately 30 min, the mice were allowed access to an unused, pre-weighed food pellet in a clean test cage containing fresh wood-chip bedding, which was placed directly under a bright light. Each mouse was placed in a corner of the test cage, and a stopwatch was immediately started. The latency to eat (s), defined as the mouse sitting on its haunches and biting the pellet with the use of forepaws, was recorded. The amount of food (g) consumed by the mouse in 5 min was measured, serving as a control for change in appetite as a possible confounding factor. The NSF assay was conducted with two individual cohorts of mice, one at 3 weeks (n = 6 per group) and another at both 9 and 11 weeks (n = 6 Veh, n = 7 PAC) post-paclitaxel (8 mg/kg, i.p.) or vehicle injection. These specific cohorts were used for both the nesting and NSF assays, since nesting is not thought to be a stress-inducing task. The mice had a resting period of one week between assays.

2.6.3. Light/dark box (LDB) test

The light/dark box test is based upon a conflict between the innate aversion to brightly illuminated areas and spontaneous exploratory activity (Crawley and Goodwin, 1980). The test was adapted as previously described (Wilkerson et al., 2016) with minor modifications. Briefly, the LDB apparatus consisted of a small, enclosed dark box (36 × 10 × 34 cm) with a passage way (6 × 6 cm) leading to a larger, light box (36 × 21 × 34 cm). The mice were acclimated to the testing room for 30 min prior to testing. Mice were placed in the light compartment and allowed to explore the apparatus for 5 min. The number of entries into the light compartment and the total time spent (s) in the light compartment were recorded for 5 min by a video monitoring system and measured by ANY-MAZE software (Stoelting Co., Wood Dale, IL). Individual cohorts of mice (n = 6 per group) were tested at 3, 6, and 9 weeks post-paclitaxel (8 mg/kg, i.p.) or vehicle injection.

2.6.4. Forced swim test (FST)

The forced swim test was performed as described previously by Damaj et al., (2004), the common method for assessing depression-like behavior in mice (Bogdanova et al., 2013). Briefly, mice were gently placed into individual glass cylinders (25 × 10 cm) containing 10 cm of water, maintained at 24 °C, and left for 6 min. Immobility was recorded (s) during the last 4 min. A mouse was considered to be immobile when floating in an upright position and only making small movements to keep its head above water, but not producing displacements. An individual cohort of mice (n = 6 per group) was tested throughout the FST study at 1, 2, 3, and 4 weeks post-paclitaxel (8 mg/kg, i.p) or vehicle injection.

2.6.5. Sucrose preference

The sucrose preference test is used as a measure of anhedonia-

like behavior (Thompson and Grant, 1971). Mice had access to two, 25 ml sipper tubes, one containing normal drinking water and the other containing a 2% sucrose solution. Mice were housed individually, with access to food, water, and 2% sucrose 24 h per day. Mice were acclimated to the cages with sipper tubes for 3 days prior to injection (days 1–3), during which baseline measurements were taken. Paclitaxel (8 mg/kg, i.p.) or vehicle injections started on day 4. Water and sucrose intake were measured on days 1, 2, 3, 4, 5, and 6, as well as on days 10, 11 and 12. The location of both sipper tubes was switched daily to avoid place preference. Sucrose preference was calculated as a percentage of the volume of 2% sucrose consumed over the total fluid intake volume. An individual cohort of mice (n = 8 per group) was tested during the vehicle/paclitaxel treatment.

2.7. Statistical analyses

In the current study, a power analysis calculation was performed with the Lamorte's Power Calculator (Boston University Research Compliance) to determine the sample size of animals for each group (Charan and Kantharia, 2013). For assessing the nociceptive behaviors, the calculation showed that an n of 5 was required to achieve a power of 90% with an alpha error of 0.05; we used 6 mice per group. For the behavioral assays, the calculations showed that an n of 5 for novelty suppressed feeding, an n of 5 for nesting, an n of 8 for the light/dark box test, an n of 6 for the forced swim test, and an n of 8 for sucrose preference was required to achieve a power of 90% with an alpha error of 0.05; we used 6 to 8 mice per group. The data were analyzed with GraphPad Prism software, version 6 (GraphPad Software, Inc., La Jolla, CA) and are expressed as mean ± SEM. Before conducting statistical analyses, normality and variance tests were performed; normality of residuals was determined by the Shapiro-Wilk test for $n > 6$ or the Kolmogorov-Smirnov test for $n \leq 6$, and equal variance was determined by the F test. Data that did not pass the normality test were analyzed by non-parametric tests, and data that did not have equal variance were analyzed without the assumption of equal standard deviations. Data were normalized to initial vehicle measurements when appropriate. Unpaired *t* tests were performed to compare behaviors of vehicle- and paclitaxel-treated mice at a single time point. Two-way repeated measure analysis of variance (ANOVA) tests were conducted, and followed by the Bonferroni post hoc test, when behavioral outcomes of vehicle- and paclitaxel-treated mice were being compared over multiple time points. Differences were considered to be significant at $P < 0.05$.

3. Results

3.1. Paclitaxel induced changes in nociceptive behaviors in mice

Initial experiments determined the effect of paclitaxel on the development of mechanical and cold allodynia as a function of the drug dose. As anticipated, increased nociceptive responses and duration of effects were related to dose of paclitaxel. However, no significant changes in body weight gain or spontaneous activity were observed. As seen in Fig. 1A and B, paclitaxel induced both mechanical allodynia [$F_{\text{dose x time}}(21, 105) = 9.481$, $P < 0.0001$] and cold allodynia [$F_{\text{dose x time}}(9, 45) = 14.76$, $P < 0.0001$] in dose- and time-related manners, respectively. At 8 mg/kg paclitaxel, mechanical allodynia was observed on day 1 post-paclitaxel injection, and this effect was sustained for more than 90 days (data not shown). On the other hand, 2 and 4 mg/kg paclitaxel induced mechanical allodynia beginning on day 3, and the effects did not differ in terms of magnitude or time to recover. With regard to cold allodynia, paclitaxel presented a clear dose-dependent induction on day 8 post-paclitaxel injection. However, mice that received 2 or 4 mg/kg paclitaxel recovered by day 22, whereas the 8 mg/kg group continued to exhibit cold allodynia. In regards to general body condition, even the highest dose of paclitaxel (8 mg/kg) did not significantly alter body weight [$F_{\text{dose x time}}(5, 25) = 1.093$, $P > 0.05$; Supplementary Fig. 1A], or motor coordination [$F_{\text{dose x time}}(4, 40) = 0.5204$, $P > 0.05$; Supplementary Fig. 1B].

3.1.1. Paclitaxel decreased the density of intra-epidermal nerve fibers (IENFs)

Because changes in the density of peripheral nerve fibers represent a hallmark of CIPN, we studied the changes in peripheral nerve fiber density following paclitaxel treatment using immunohistochemistry. At 28 days post-paclitaxel injection, mice treated with paclitaxel (8 mg/kg, 1 cycle) demonstrated significant reductions in the density of IENFs when compared to vehicle-treated mice [$t = 3.736$, df = 10, $P < 0.01$; Fig. 2A]. Representative immunostained sections of foot pads from vehicle- (Fig. 2B; upper panel) and paclitaxel-treated mice (Fig. 2B; lower panel) show the reduction in IENFs following paclitaxel treatment.

3.1.2. Impact of repeated drug cycles on paclitaxel-induced mechanical allodynia

To investigate the effect of repeated cycles of paclitaxel on mechanical allodynia, mice were injected with two cycles of a low dose of paclitaxel (2 mg/kg). As expected, the first cycle of paclitaxel (2 mg/kg) was capable of inducing mechanical allodynia. Indeed, paclitaxel (2 mg/kg) induced a significant reduction in mechanical threshold that lasted for at least 14 days after the first injection of paclitaxel [$F_{\text{dose x time}}(7, 35) = 8.436$, $P < 0.0001$; Fig. 3A]. After a one week resting period, mice received another cycle of paclitaxel (2 mg/kg). Surprisingly, the effects of paclitaxel were significantly enhanced in the mice subjected to a second cycle, which was demonstrated by a further decrease in mechanical threshold [$F_{\text{dose x time}}(3, 15) = 48.61$, $P < 0.0001$; Supplementary Fig. 2]. In addition, mice that received a second cycle of paclitaxel treatment (2 mg/kg) displayed a much longer duration of allodynia (Fig. 3B) compared to one cycle of treatment (Fig. 3A) [$F_{\text{dose x time}}(13, 65) = 10.97$, $P < 0.0001$; Fig. 3B]. Whereas mice given one cycle recovered by day 21 post-paclitaxel injection, mice given two cycles recovered by day 63 after the first injection of paclitaxel. Calculation of the area under the curve (AUC) threshold for the initial 28 days of both the first and second cycles of paclitaxel treatment revealed significant differences (2.5 fold difference) between cycles [$F_{treatment}(3, 20) = 60.35$, $P < 0.0001$; Fig. 3C].

3.1.3. Paclitaxel induced allodynia following carboplatin treatment

We further investigated the impact of carboplatin treatment on paclitaxel-induced allodynia. Mice given one cycle of carboplatin alone did not demonstrate significant mechanical nociceptive changes. As shown in Supplementary Fig. 3, one cycle of 5 or 20 mg/kg carboplatin did not significantly reduce the mechanical threshold [$F_{\text{dose x time}}(8, 40) = 0.4526$, $P > 0.05$]. However, in a separate cohort of mice, a low-dose paclitaxel (1 mg/kg) cycle administered one week following the completion of the carboplatin (5 mg/kg) cycle led to a significant reduction of mechanical threshold compared to the vehicle-paclitaxel group [$F_{\text{dose x time}}(12, 60) = 16.65$, $P < 0.0001$; Fig. 3D].

3.2. Paclitaxel induced changes in affective-related behaviors in mice

To assess whether paclitaxel interferes with the natural behavior of mice, a nesting assay was conducted at various time points after paclitaxel treatment was initiated. However, paclitaxel

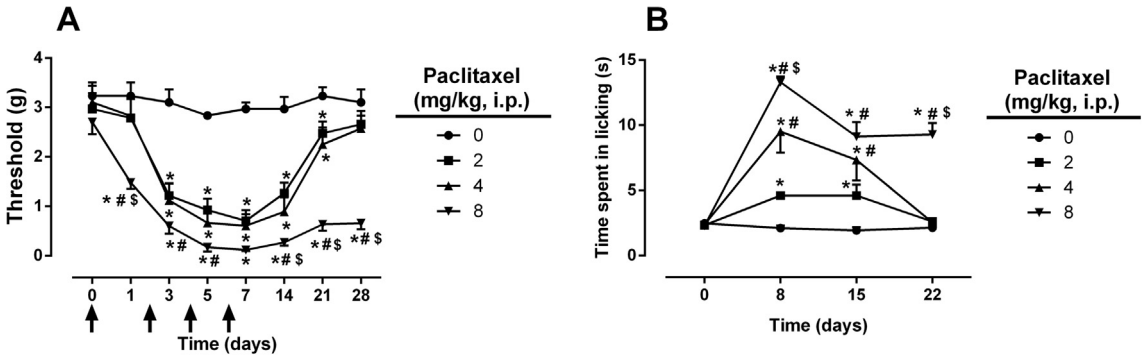

Fig. 1. Paclitaxel induces nociceptive behaviors. Paclitaxel doses of 2, 4, and 8 mg/kg (i.p., every other day for a total of 4 injections) induce both mechanical (A) and cold (B) allodynia in a dose and time dependent manner. Arrows indicate vehicle/paclitaxel injections on days 0, 2, 4, and 6. Baseline measurements were taken before vehicle/paclitaxel administration on day 0. The same cohort was tested for both mechanical and cold allodynia; n = 6 per group (data expressed as mean ± SEM). $^{*}P < 0.05$ vs vehicle; $^{\#}P < 0.05$ vs paclitaxel (2 mg/kg); $^{\$}P < 0.05$ vs paclitaxel (4 mg/kg).

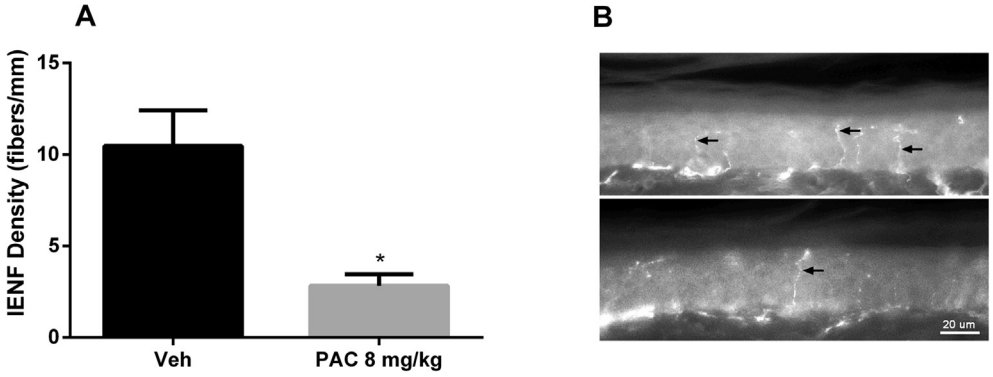

Fig. 2. Paclitaxel induces a reduction in intra-epidermal nerve fiber (IENF) density at 28 days post-paclitaxel injection. A) Quantification of IENF density in mice treated with one cycle of paclitaxel (8 mg/kg, i.p., every other day for a total of 4 injections) shows a significant reduction compared to vehicle. One cohort was tested; n = 6 per group (data expressed as mean ± SEM). $^{*}P < 0.05$ vs vehicle. Veh, vehicle; PAC, paclitaxel. B) Immunostained sections of vehicle- (upper panel) and paclitaxel-treated (lower panel) hind foot pad skin showing the reduction of IENFs (arrows) following paclitaxel treatment. Bar represents 20 microns in both images.

did not interfere with nesting activity [z = 0.856, $P > 0.05$; $z = 1.000$, $P > 0.05$], the quantity of nesting material used [$t = 0.08655$, df = 10, $P > 0.05$; $t = 0.03402$, df = 10, $P > 0.05$], or nest quality [$t = 0.4152$, df = 10, $P > 0.05$; $t = 0.2033$, df = 10, $P > 0.05$] at 1 and 2 weeks post-paclitaxel injection, respectively (Fig. 4). Similar results were observed at 8 and 11 weeks post-paclitaxel injection, in which nesting activity was not significantly affected by paclitaxel [z = 0.926, $P > 0.05$; Fig. 4A]. The use of nesting material [$F_{\text{treatment x time}}$ (1,11) = 1.157, $P > 0.05$] and nest quality [$F_{\text{treatment x time}}$ (1,11) = 0.0094, $P > 0.05$] were also not found to be significantly altered (Fig. 4B and C).

With regard to affective-related changes, we assessed anxiety-, depression-, and anhedonia-like behaviors at various time points in mice treated with paclitaxel, according to the aforementioned treatment regimen. Alterations in anxiety were assessed utilizing the novelty suppressed feeding (NSF) assay. Paclitaxel significantly increased the latency to eat in a novel environment at 3 and 9 weeks post-paclitaxel injection (Fig. 5A). A significant increase in latency to eat occurred at 3 weeks post-paclitaxel treatment [$t = 2.224$, df = 12, $P < 0.05$, Fig. 5A]. In addition, significant differences in latency to eat between vehicle- and paclitaxel-treated mice occurred at 9 weeks post-paclitaxel injection ($P < 0.05$), which dissipated by week 11. The amount of food consumed in the test cage was not impacted by paclitaxel treatment (Fig. 5B).

Paclitaxel was also found to induce anxiety-like behavior in the light/dark box (LDB) test, in which time spent in the light compartment of the LDB apparatus was significantly decreased at 3 weeks [$t = 2.277$, df = 14, $P < 0.05$], 6 weeks [$t = 2.350$, df = 14, $P < 0.05$], and 9 weeks [$t = 2.309$, df = 14, $P < 0.05$] post-paclitaxel treatment (Fig. 6). Importantly, the *number* of entries into the light compartment was not significantly decreased at any time point for the paclitaxel-treated mice (Table 1), suggesting that the decrease in time spent in the light compartment is not due to motor deficits (Supplementary Fig. 1B).

The mice were then evaluated for depression-like behavior in FST, an experimental paradigm that assesses immobility when placed in a container of water. Within the same cohort of mice, paclitaxel treatment induced an emotional-like deficit during FST [$F_{\text{treatment x time}}$ (3,15) = 6.200, $P < 0.01$; Fig. 7]. The time spent immobile during FST was significantly increased at 2 and 3 weeks post paclitaxel-injection ($P < 0.01$), an effect that dissipated by week 4 (Fig. 7).

Lastly, anhedonia-like behavior was assessed using the sucrose preference test. The interaction between paclitaxel treatment and time was significant within the same cohort of mice [$F_{\text{treatment x time}}$ (8,112) = 9.424, $P < 0.0001$, Fig. 8]. Paclitaxel produced a significant decrease in sucrose preference during ($P < 0.0001$) and shortly after ($P < 0.01$, $P < 0.05$) completion of the treatment regimen when compared to vehicle-treated mice (Fig. 8). To ensure that the decrease in consumatory behavior was not due to a decrease in overall consumption, we assessed total fluid intake between vehicle- and paclitaxel-treated mice, which was found to not differ significantly between the two groups (Supplementary Fig. 4).

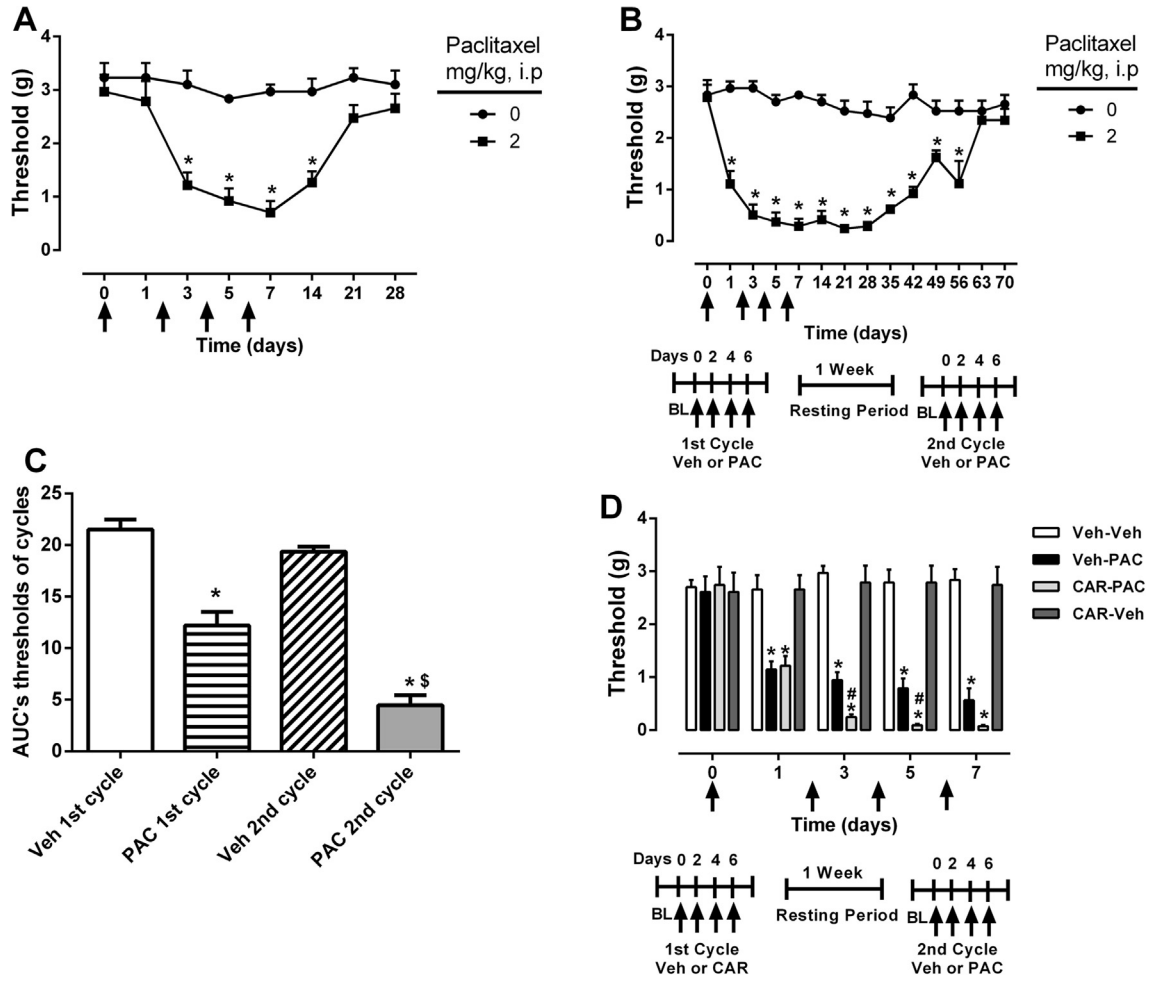

Fig. 3. Mice are sensitized to cutaneous stimulation after second cycle of paclitaxel treatment. A) Mice treated with one cycle of paclitaxel (2 mg/kg) or vehicle (i.p., every other day for a total of 4 injections). B) Mice from 3A treated with a second cycle of paclitaxel (2 mg/kg) or vehicle (i.p., every other day for a total of 4 injections). C) AUC mechanical threshold for initial 28 days of first and second cycles of paclitaxel treatment. D) Comparison of mechanical thresholds during the second cycle of treatment between mice treated with carboplatin (5 mg/kg) alone and with carboplatin followed by a low dose of paclitaxel (1 mg/kg). Arrows indicate vehicle/paclitaxel/carboplatin injections on days 0, 2, 4, and 6 of each cycle. Baseline measurements were taken before vehicle/paclitaxel/carboplatin administration on day 0. One cohort was tested; n = 6 per group (data expressed as mean ± SEM). $^{*}P < 0.05$ vs vehicle; $^{\$}P < 0.05$ vs first cycle of paclitaxel (2 mg/kg), $^{\#}P < 0.05$ vs carboplatin (5 mg/kg). BL, baseline; Veh, vehicle; PAC, paclitaxel; CAR, carboplatin.

4. Discussion

The results of the present study demonstrate that a clinically relevant dosing regimen of paclitaxel given systemically to male C57BL/6J mice causes the induction and long-term maintenance of mechanical and cold allodynia, as well as negative affective-related symptoms, including anxiety- and depression-like behaviors of shorter duration. These changes occurred without significant decreases in body weight or impairment of locomotion following paclitaxel treatment (Supplementary Fig. 1), findings that are in accordance with other studies showing that various doses of paclitaxel do not alter body weight (Boehmerle et al., 2014) or locomotor activity (Deng et al., 2015; Neito et al., 2008).

Few studies have been performed under similar experimental conditions examining the effect of various doses of paclitaxel on the development of mechanical and cold allodynia, especially during the early period of injection and regarding the magnitude of that allodynia. Our results are consistent with other reports showing that paclitaxel induces both mechanical and cold allodynia in male mice (Deng et al., 2015; Slivicki et al., 2016; Naji-Esfahani et al., 2016). Interestingly, Ward et al., (2011) reported that a cycle of low-dose paclitaxel (1 or 2 mg/kg) elicited a considerably greater magnitude of cold allodynia in female mice than in male mice. Importantly, it has been noted in the clinic that neuropathic pain is more prevelant in women than in men (Fillingim et al., 2009). Therefore, it is possible that sex differences may arise in affective-like behaviors, along with nociceptive behaviors, following paclitaxel treatment.

With regard to morphological changes, our experiments show that 8 mg/kg paclitaxel produces a robust decrease in the density of intra-epidermal nerve fibers (IENFs), which is consistent with the results of Krukowski et al. (2015) that demonstrate significant reductions in IENF density following repeated administrations of paclitaxel in mice. Additional studies in rats have shown a dose-dependent decrease in IENFs following a wide range of paclitaxel doses (0.5–32 mg/kg), as well as a correlation between paclitaxel-induced loss of IENFs and allodynia (Bennett et al., 2011; Ko et al., 2014). Also, it is known that the polymodal C and Aδ fibers are retracted following paclitaxel administration (Basbaum et al., 2009; Landowski et al., 2016; Vichaya et al., 2015). Despite the decrease in IENF density, the remaining nociceptive fibers can become hyperactive and/or sensitized due to their release of chemical mediators of inflammation, such as substance P and calcitonin gene-related peptide (CGRP), as well as exposure to pro-inflammatory

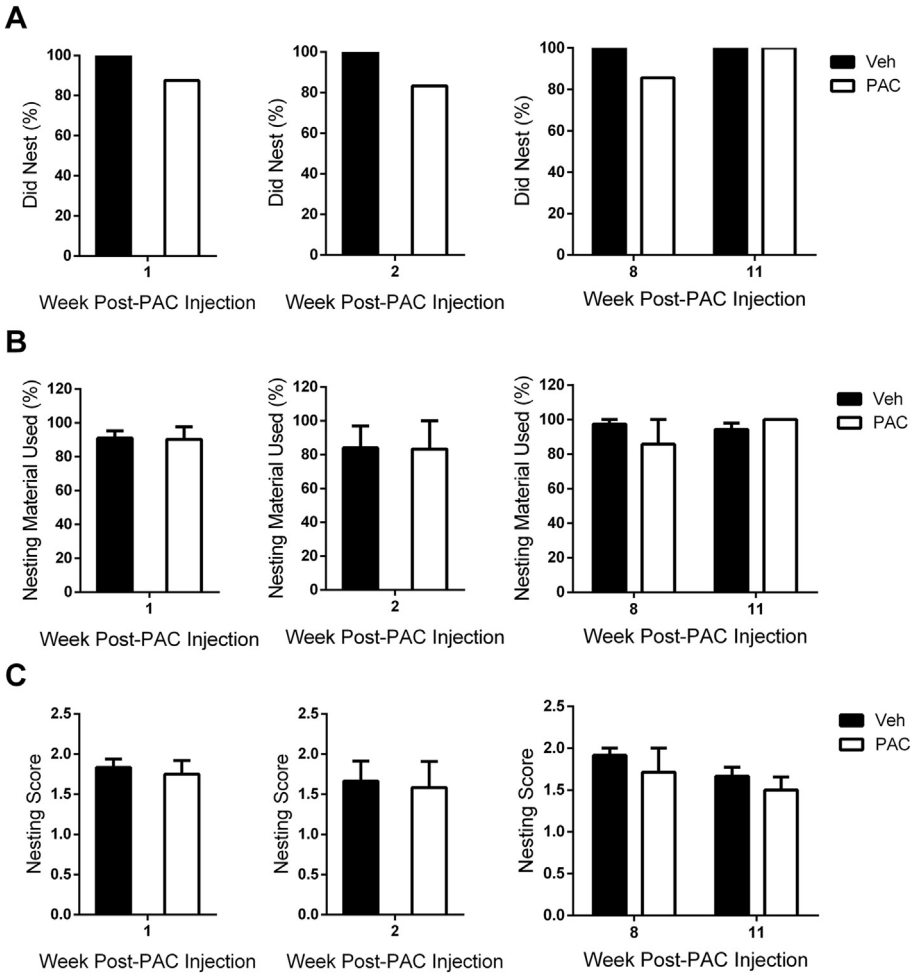

Fig. 4. Paclitaxel does not influence the nesting behavior of mice. Mice were allowed 120 min to nest at weeks 1, 2, 8, and 11 post-paclitaxel (8 mg/kg, i.p.) or vehicle injection. A) It was determined that mice had participated in nesting activity if at least one nestlet piece had been chewed or pulled into the home cage. A comparison of proportions via z-tests between vehicle- and paclitaxel-treated mice was not significant at any time point. B) The percentage of nesting material used was determined by the following equation: (weight of initial nestlet pieces − weight of remaining nestlet pieces)/weight of initial nestlet pieces. C) The quality of each nest was evaluated on a scale ranging from 0 to 2 (0 = no nest formed, 1 = some nesting activity, 2 = established nest). Individual cohorts were tested at 1 week (n = 6 per group), 2 weeks (n = 6 per group), 8 and 11 weeks (n = 6 Veh, n = 7 PAC) post-paclitaxel (8 mg/kg, i.p.) or vehicle injection; data expressed as mean ± SEM. Post-PAC injection refers to the time following the first of four paclitaxel injections. Veh, vehicle; PAC, paclitaxel.

cytokines released by infiltrating immune cells, such as macrophages (Carozzi et al., 2015).

The present study also revealed that two cycles of 2 mg/kg paclitaxel (cumulative dose of 16 mg/kg) causes mice to exhibit lower mechanical thresholds than mice that received the same cumulative dose following one cycle of 4 mg/kg paclitaxel (Supplementary Fig. 2; Fig. 1A). This finding suggests that sensitization occurs during the first cycle of paclitaxel treatment. The observed sensitization may be due to the accumulation of paclitaxel in the periphery, as detectable concentrations of paclitaxel have been measured in the dorsal root ganglia and the sciatic nerve up to 26 days post-paclitaxel dosing (Wozniak et al., 2016).

In the clinic, paclitaxel has been administered in combination with cisplatin in non-small cell lung cancer patients. The combination produces additional neurotoxicity, and even two cycles of the treatment can result in neuropathy (Arrieta et al., 2010). In an attempt to avoid this toxicity, paclitaxel and carboplatin have been used in combination. Carboplatin is considered to be less neurotoxic than cisplatin and only 4–6% of patients who receive carboplatin may develop peripheral neuropathy (McWhinney et al., 2009). Furthermore, a study in ovarian cancer patients revealed that the carboplatin-paclitaxel treatment induced significantly less peripheral neuropathy than that produced by the cisplatin-paclitaxel treatment (Neijt et al., 2000). Clinical studies have shown that administration of carboplatin before paclitaxel is feasible in patients (Markman et al., 2003; Davidson et al., 2016). Our data show that in contrast to paclitaxel, mice treated with carboplatin (5 or 20 mg/kg) alone failed to show significant allodynia. However, when a low dose of carboplatin (5 mg/kg) was followed by a low dose of paclitaxel (1 mg/kg), mice develop more severe mechanical allodynia when compared to paclitaxel alone, suggesting that carboplatin sensitized the mice to subsequent paclitaxel treatment. To our knowledge, studies of carboplatin- or carboplatin-paclitaxel-induced mechanical allodynia in mice have not been reported previously.

This work also investigated the affective-related consequences of paclitaxel treatment. Using a paclitaxel regimen that caused a long-lasting allodynia (8 mg/kg, 1 cycle), we observed an increase in the latency to eat during the NSF assay and aversion to the light compartment of the LDB apparatus. These effects in two tests of anxiety suggest that, under the present experimental conditions, paclitaxel induces an anxiety-like state. We also found that

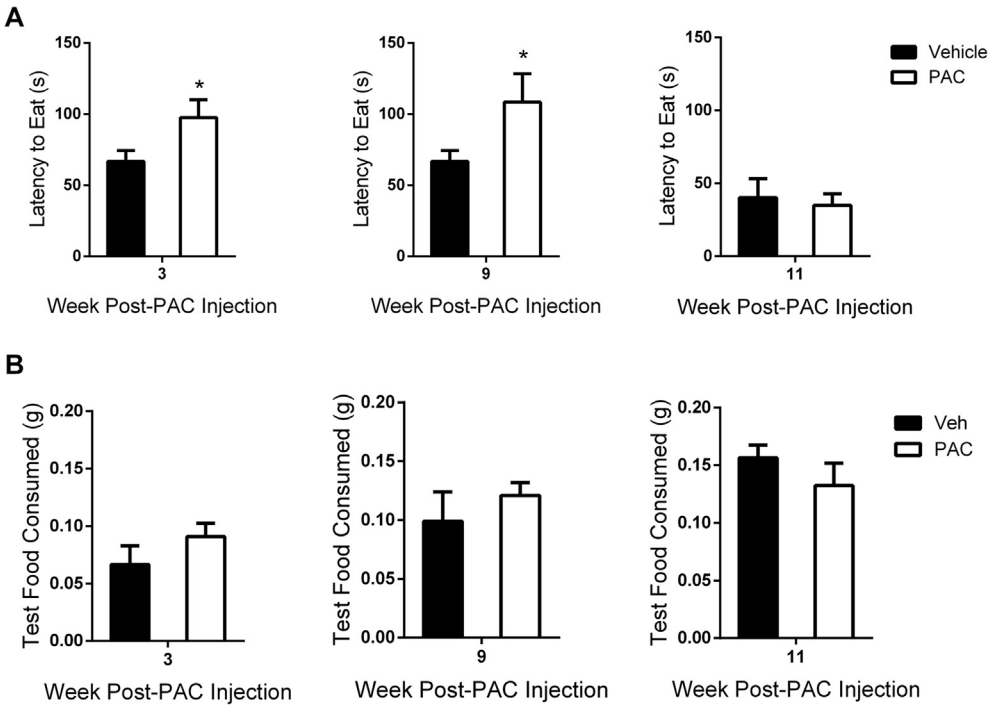

Fig. 5. Paclitaxel induces anxiety-like behavior in the novelty suppressed feeding assay. (A) Latency to eat test cage food was determined as the time in seconds from when the mouse was placed inside the test cage until the mouse sat on its haunches while holding and biting the food pellet. (B) Consumption of test cage food was calculated with the following equation: (initial weight of food pellet − weight of food pellet after 5 min eating period in test cage). Individual cohorts were tested at 3 weeks (n = 6 per group), 9 and 11 weeks (n = 6 Veh, n = 7 PAC) post-paclitaxel (8 mg/kg, i.p.) or vehicle injection; data expressed as mean ± SEM. *$P < 0.05$ vs vehicle. Post-PAC injection refers to the time following the first of four paclitaxel injections. Veh, vehicle; PAC, paclitaxel.

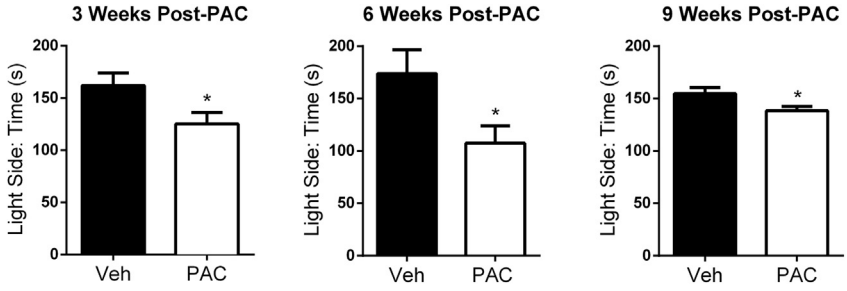

Fig. 6. Paclitaxel induces anxiety-like behavior in the light/dark box test. Mice were free to explore both light and dark compartments for 5 min. The study was conducted with individual cohorts of mice (n = 8 per group) at 3, 6, and 9 weeks post-paclitaxel (8 mg/kg, i.p.) or vehicle injection; data expressed as mean ± SEM. *$P < 0.05$ vs vehicle. Post-PAC injection refers to the time following the first of four paclitaxel injections. Veh, vehicle; PAC, paclitaxel.

Table 1
Paclitaxel treatment does not interfere with entry into the light compartment of the light/dark box apparatus. Unpaired *t* tests revealed no significant differences between vehicle- and paclitaxel-treated mice at any time point. One experiment was conducted with individual cohorts of mice (n = 8 per group) at each time point. Post-PAC injection refers to the time following the first of four paclitaxel injections. Data are expressed as mean ± SEM.

Light Side: Number of Entries			
	3 Weeks Post-PAC	6 Weeks Post-PAC	9 Weeks Post-PAC
Vehicle	16 ± 1.7	15 ± 2.0	14 ± 1.7
Paclitaxel	14 ± 1.9	13 ± 1.9	12 ± 1.7

paclitaxel-treated mice exhibit increased immobility time during FST and anhedonia-like behavior in the sucrose preference test. The observed decrease in sucrose preference could also indicate that an alteration in taste (dysgeusia), a phenomenon seen in some patients receiving paclitaxel (Turcott et al., 2016), is occurring during paclitaxel treatment; yet, we cannot make that conclusion from a single oral consumption assay. The possible taste alteration may produce decreased appetite, but no significant changes in body weight were detected. Collectively, these results indicate that in addition to peripheral neuropathy signs, paclitaxel induces a deficit in the emotional-like state of the mice. Conversely, paclitaxel did not affect nesting behavior, an assay that has been shown to reflect pain-depressed behavior when lactic acid and complete Freund adjuvant (CFA) are used as noxious stimuli (Negus et al., 2015). The lack of an effect in this assay is consistent with the hypothesis that the value of a habit-like survival task does not alter depending on the motivational state (Rock et al., 2014). Thus, the necessity of establishing a nest for thermoregulation, fitness, and shelter may overcome the nociceptive and negative affective symptoms of paclitaxel.

To increase our understanding of paclitaxel-induced toxicity, the

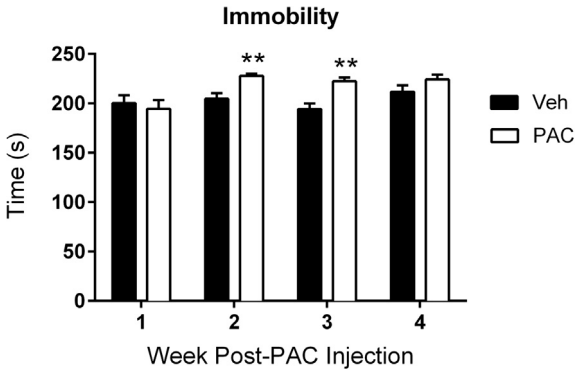

Fig. 7. Paclitaxel induces depression-like behavior in the forced swim test. Time represents the number of seconds the mouse was immobile when placed in water; the cut-off time was 240 s. The same cohort of mice (n = 6 per group) was tested at weeks 1, 2, 3, and 4 post-paclitaxel (8 mg/kg, i.p.) or vehicle injection; data expressed as mean ± SEM. $^{**}P < 0.01$ vs vehicle. Post-PAC injection refers to the time following the first of four paclitaxel injections. Veh, vehicle; PAC, paclitaxel.

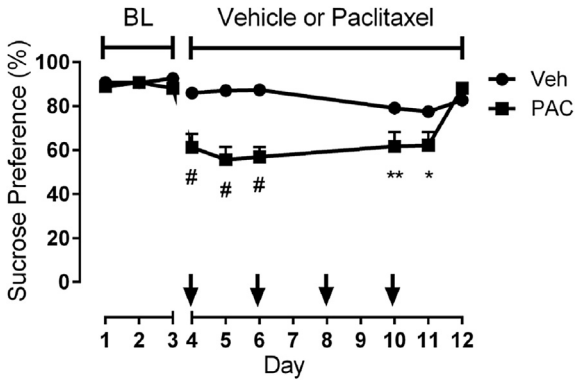

Fig. 8. Paclitaxel induces anhedonia-like behavior in the sucrose preference test. Mice were provided with two sipper tubes, one containing normal drinking water and the other containing a 2% sucrose solution, for 24 h per day. Sucrose preference was determined as the percentage of 2% sucrose volume consumed over the total fluid intake volume. Arrows indicate the time of each paclitaxel (8 mg/kg, i.p.) or vehicle injection. The study was conducted with the same cohort of mice (n = 8 per group) during paclitaxel (8 mg/kg, i.p.) or vehicle injections; data expressed as mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{\#}P < 0.0001$ vs vehicle. BL, baseline; Veh, vehicle; PAC, paclitaxel.

relationship between nociceptive and affective symptoms needs to be considered, as well as the temporal order in which these side effects develop. Studies have shown that the pathology of a tumor itself can cause emotional disturbances in rodents (Pyter et al., 2009), but our experiments in non-tumor-bearing mice reveal that paclitaxel alone is also capable of inducing anxiety- and depression-like behaviors. At 1 week post-paclitaxel injection, we observed the development of both mechanical and cold allodynia, as well as anhedonia-like behavior (Table 2). Anxiety- and depression-like behaviors arise in the subsequent weeks following paclitaxel treatment. The immediate appearance of nociceptive symptoms is consistent with paclitaxel acting directly on the peripheral nervous system, but there may be a separate central mechanism of the drug. While paclitaxel seems to accumulate in peripheral organs such as the peripheral nervous system, it has been detected in the brain of mice following tail vein injection, even at low concentrations (Gangloff et al., 2005; Kemper et al., 2003), suggesting that it crossed the blood brain barrier. Therefore, the presence of paclitaxel in the central nervous system and/or paclitaxel-induced peripheral neuropathy itself may be causing changes in affective behaviors through neuroinflammation

Table 2
Summary of onset and duration of nociceptive, natural, and affective behaviors. Post-PAC injection refers to the time following the first of four paclitaxel injections. NSF, novelty suppressed feeding; LDB, light/dark box; FST, forced swim test; (−), no phenotype; (+), nociceptive/affective behavior; ND, not determined.

Behavior	Assay	Weeks Post-PAC Injection					
		1	2–3	4–5	6–7	8–9	10–11
Nociceptive	Mechanical Allodynia	+	+	+	+	+	+
	Cold Allodynia	+	+	ND	ND	ND	ND
Natural	Nesting	−	−	ND	ND	−	−
Anxiety-like	NSF	ND	+	ND	ND	+	−
	LDB	ND	+	ND	+	+	ND
Depression-like	FST	−	+	−	ND	ND	ND
	Sucrose Preference	+	−	ND	ND	ND	ND

mechanisms and/or an induction of central neurotoxicity. It is also possible that paclitaxel-induced sensitization of immune responses may have played a role in the development of peripheral neuropathy, and perhaps of affective-like behaviors. Indeed, hypersensitivity to stimuli, not only in neuropathic pain but also in inflammatory pain, can be explained by both peripheral and central sensitization of sensory nerve fibers (Fornasari, 2012). In regards to the neuroimmune interface, glial responses have also been shown to play a role in central and peripheral nervous system function during neuropathic pain (Scholz and Woolf, 2007).

The differences between the onset, duration, and resolution of these affective behaviors should also be considered. Although changes in nociceptive behavior, such as mechanical allodynia, occur immediately following paclitaxel administration, there appears to be a delay in the initiation of emotional-like deficits. Clinically, somatic and affective symptoms can occur simultaneously. Breast cancer patients often experience a cluster of symptoms including pain (77%), anxiety (21%), and depression (36%), indicating that they may share a common mechanism (So et al., 2009). Those patients receiving chemotherapy experience the cluster symptoms to a greater degree and are at a higher risk for decreased quality of life.

The time-dependent development of both anxiety- and depression-like behaviors has also been observed in other mouse neuropathic pain models. La Porta et al., (2016) reported ipsilateral mechanical and cold allodynia from day 3 to day 27 post-partial sciatic nerve ligation (PSNL) in Swiss albino male mice, with enhanced anxiety-like behavior in the elevated plus maze from 1 to 3 weeks post-PSNL and increased depressive-like behavior during FST, but only at 3 weeks post-PSNL. Also, a significant decrease in sucrose preference was observed from day 1 to day 20 post-PSNL. Although this study utilized a different model of neuropathic pain, alterations in nociceptive behaviors were also induced immediately and persisted for approximately four weeks. However, we found that anxiety-like behavior can be maintained for 9 weeks following nerve exposure to a noxious stimulus. Consistent findings were made in regards to depression-like behavior, in which increased immobility during FST did not appear until 2–3 weeks. We recognize that repeated testing of the same cohort during FST could be a limitation, however, vehicle-treated mice did not express adaptation to the assay. The development of anhedonia-like behavior was also similar, during which a decrease in sucrose preference was observed the day following PSNL or paclitaxel treatment, but the effect only persisted for 11 days post-paclitaxel injection, whereas PSNL induced this behavior until day 20.

Similarly, using sciatic nerve constriction (SNC) in male C57BL/6J mice, Yalcin et al., (2011) reported that ipsilateral mechanical allodynia persisted for 90 days, and increased anxiety-like behavior in the light/dark box test was observed at 4, 7, and 8 weeks post-

SNC, a time-dependent effect similar to that seen in the present study. Latency to first contact and bite the food pellet during the NSF assay was observed at 5 and 8 weeks post-SNC, an effect that appeared earlier in paclitaxel-induced neuropathic pain. Increased immobility in neuropathic mice was observed at 8 and 9 weeks post-SNC during FST, whereas paclitaxel-induced neuropathic pain caused immobility at 2 and 3 weeks post-paclitaxel injection. The differences and similarities amongst these studies illustrate the importance of establishing a clinically relevant model specific to the type of neuropathic pain of interest in order to best determine the responsible mechanisms. Also, these data suggest that multiple pathways and/or brain regions are involved in the manifestation of affective-related behaviors. Yet it remains plausible that paclitaxel administration and models of nerve injury share common mechanisms for the induction of affective-related behaviors.

In conclusion, this work characterizes a preclinical mouse model of both the nociceptive and negative affective symptoms of paclitaxel treatment, which can be utilized to test the efficacy of potential therapeutics for the treatment of paclitaxel-induced side effects, as well as investigate mechanisms of action. In addition, this study allows for the separate investigation of chemotherapy-induced pain-related behaviors in a tumor-free environment, which cannot be ethically accomplished in a clinical setting.

Conflicts of interest

The authors declare no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.neuropharm.2017.02.020.

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Review

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Molecular definitions of autophagy and related processes

Lorenzo Galluzzi[1,2,*], Eric H Baehrecke[3], Andrea Ballabio[4,5,6,7], Patricia Boya[8], José Manuel Bravo-San Pedro[2,9,10,11,12], Francesco Cecconi[13,14,15], Augustine M Choi[16], Charleen T Chu[17], Patrice Codogno[2,18,19,20], Maria Isabel Colombo[21,22], Ana Maria Cuervo[23], Jayanta Debnath[24], Vojo Deretic[25], Ivan Dikic[26,27,28], Eeva-Liisa Eskelinen[29], Gian Maria Fimia[30,31], Simone Fulda[32,33,34], David A Gewirtz[35,36], Douglas R Green[37], Malene Hansen[38], J Wade Harper[39], Marja Jäättelä[40], Terje Johansen[41], Gabor Juhasz[42,43], Alec C Kimmelman[44], Claudine Kraft[45], Nicholas T Ktistakis[46], Sharad Kumar[47], Beth Levine[48,49], Carlos Lopez-Otin[50,51], Frank Madeo[52,53], Sascha Martens[45], Jennifer Martinez[54], Alicia Melendez[55,56], Noboru Mizushima[57], Christian Münz[58], Leon O Murphy[59], Josef M Penninger[60], Mauro Piacentini[13,30], Fulvio Reggiori[61], David C Rubinsztein[62], Kevin M Ryan[63], Laura Santambrogio[64], Luca Scorrano[65,66], Anna Katharina Simon[67,68], Hans-Uwe Simon[69], Anne Simonsen[70], Nektarios Tavernarakis[71,72], Sharon A Tooze[73], Tamotsu Yoshimori[74,75], Junying Yuan[76,77], Zhenyu Yue[78], Qing Zhong[48] & Guido Kroemer[2,9,10,11,12,79,80]

Abstract

Over the past two decades, the molecular machinery that underlies autophagic responses has been characterized with ever increasing precision in multiple model organisms. Moreover, it has become clear that autophagy and autophagy-related processes have profound implications for human pathophysiology. However, considerable confusion persists about the use of appropriate terms to indicate specific types of autophagy and some components of the autophagy machinery, which may have detrimental effects on the expansion of the field. Driven by the overt recognition of such a potential obstacle, a panel of leading experts in the field attempts here to define several autophagy-related terms based on specific biochemical features. The ultimate objective of this collaborative exchange is to formulate recommendations that facilitate the dissemination of knowledge within and outside the field of autophagy research.

Keywords chaperone-mediated autophagy; LC3-associated phagocytosis; microautophagy; mitophagy; xenophagy

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Introduction

The Nobel Assembly at Karolinska Institute awarded the 2016 Prize in Physiology or Medicine to the cell biologist Yoshinori Ohsumi for his early identification and characterization of the autophagy machinery, in particular, AuTophaGy-related (Atg) genes, in yeast (Tsukada & Ohsumi, 1993). This came as an overt recognition to a field symbolically initiated by the Belgian cytologist and biochemist Christian De Duve, who in 1963 employed the term autophagy (from the Ancient Greek αὐτόφαγος, meaning "self-eating") for describing the presence of single- or double-membraned intracellular vesicles that contain parts of the cytoplasm and organelles in various states of disintegration (Yang & Klionsky, 2010). Our understanding of autophagy, which is highly conserved during evolution (Table 1), has tremendously expanded over the past decades, on both mechanistic and pathophysiological grounds (Choi *et al*, 2013; Noda & Inagaki, 2015). In parallel, we have begun to appreciate the considerable potential of pharmacological agents or dietary interventions that activate or inhibit autophagy as novel therapies for multiple human disorders and pathophysiological conditions, including neurodegenerative (Menzies *et al*, 2015), infectious (Deretic *et al*, 2013), autoimmune (Deretic *et al*, 2013; Zhong *et al*, 2016), cardiovascular (Shirakabe *et al*, 2016), rheumatic (Rockel & Kapoor, 2016), metabolic (Kim & Lee, 2014), pulmonary (Nakahira

1–80 The list of affiliations appears at the end of this article
*Corresponding author. Tel: +1 212 746 2231; E-mail: deadoc@vodafone.it

et al, 2016), and malignant diseases (Galluzzi *et al*, 2015b, 2017a; Amaravadi *et al*, 2016), as well as aging (Melendez *et al*, 2003; Lapierre *et al*, 2015; Lopez-Otin *et al*, 2016). Nevertheless, there is not a single drug currently licensed by the US Food and Drug Administration (FDA)—or equivalent regulatory agency—that was developed with the primary aim of modulating autophagy (although many FDA-approved drugs indeed activate or inhibit autophagy to some extent) (Poklepovic & Gewirtz, 2014; Rosenfeld *et al*, 2014; Vakifahmetoglu-Norberg *et al*, 2015). Such a barrier in the translation of robust preclinical data from multiple model organisms into clinically viable therapeutic interventions reflects the persistence of several obstacles of pharmacological, biological, and technological nature. Discussing these issues in a comprehensive manner goes well beyond the scope of the current article and has been done elsewhere (Galluzzi *et al*, 2017b). An analysis of the literature also reveals considerable confusion about the use of several autophagy-related terms, affecting not only less-experienced investigators but also researchers with many years of experience in the field. Although such a semantic issue may appear trivial at first glance, we are concerned that it may constitute a significant obstacle to the optimal development of autophagy research, both at preclinical and translational levels. This problem has been overtly recognized and discussed throughout the past year. Starting from such a constructive exchange and driven by the success obtained by a similar initiative in the cell death field (Galluzzi *et al*, 2012, 2015a), leading experts in autophagy decided to gather and tentatively define several autophagy-related terms based on precise biochemical features of the process.

Processes

Autophagy

Perhaps surprisingly, the relatively broad term "autophagy" itself has been used with rather variable and sometimes misleading connotations. We agree on two main features that characterize *bona fide*, functional autophagic responses, irrespective of type: (i) they involve cytoplasmic material; and (ii) they culminate with (and strictly depend on) lysosomal degradation. Thus, although autophagy substrates (see below for a definition) can be endogenous, such as damaged mitochondria and nuclear fragments, or exogenous, such as viruses or bacteria escaping phagosomes, autophagy operates on entities that are freely accessible to cytosolic proteins (notably, components of the autophagy machinery). This feature is important in order to discriminate autophagic responses from branches of vesicular trafficking that originate at the plasma membrane, which also culminates in lysosomal degradation. Such endocytic processes (which have cumulatively been referred to as "heterophagy" in the past) include phagocytosis (i.e., the uptake of particulate material by professional phagocytes—such as macrophages and immature dendritic cells—or other cells), receptor-mediated endocytosis (i.e., the uptake of extracellular material driven by plasma membrane receptors), and pinocytosis (i.e., the relatively non-specific uptake of extracellular fluids and small molecules) (Munz, 2017; Foot *et al*, 2017). However, some forms of autophagy (notably macroautophagy and endosomal microautophagy, see below for definitions) and the endocytic pathway interact at multiple levels, and the molecular machinery responsible for the fusion of late endosomes (also known as multi-vesicular bodies) or autophagosomes (see below for a definition) with lysosomes is essentially the same (Tooze *et al*, 2014).

The strict dependency of autophagic responses on lysosomal activity is important to discriminate them from other catabolic pathways that also involve cytoplasmic material, such as proteasomal degradation (Bhattacharyya *et al*, 2014). The 26S proteasome degrades a large number of misfolded cytoplasmic proteins that have been ubiquitinated, as well as properly folded proteins that expose specific degradation signals, such as the so-called N-degrons (Sriram *et al*, 2011). When ubiquitinated proteins accumulate, however, they tend to assemble into aggregates that are degraded by macroautophagy upon binding to autophagy receptors (see below for a definition) (Lim & Yue, 2015; Moscat *et al*, 2016). Moreover, considerable cross talk between the proteasome and chaperone-mediated autophagy (CMA, see below for a definition) has been described (Massey *et al*, 2006; Schneider *et al*, 2014), and cytosolic proteins bound to heat shock protein family A (Hsp70) member 8 (HSPA8), which serves as the main chaperone in CMA, can be efficiently redirected to proteasomal degradation upon interaction with ubiquilin 2 (UBQLN2) (Hjerpe *et al*, 2016). Thus, the proteasome system shares some substrates with different forms of autophagy. However, these two catabolic pathways differ radically in their final products. Proteasomal degradation results in short peptides (of 8–12 residues) that are not necessarily degraded further, but may feed into additional processes including (but not limited to) antigen presentation at the plasma membrane (Neefjes *et al*, 2011). In contrast, lysosomal proteases fully catabolize polypeptides to their constituting amino acids, which eventually become available for metabolic reactions or repair processes. Moreover, lysosomal hydrolases also degrade lipids, sugars, and nucleic acids (Settembre *et al*, 2013). In summary, *bona fide* functional *autophagic responses* direct cytoplasmic material of endogenous or exogenous origin to degradation within lysosomes (or late endosomes, in specific cases).

Microautophagy and endosomal microautophagy

Microautophagy is a form of autophagy during which cytoplasmic entities destined for degradation are directly taken up by the vacuole (in yeast and plants) via direct membrane invagination (Farre & Subramani, 2004; Uttenweiler & Mayer, 2008). In cells from *Drosophila melanogaster* and mammals, a similar mechanism involves late endosomes. This process, which also occurs in yeast cells, is commonly known as "endosomal microautophagy" (Sahu *et al*, 2011; Uytterhoeven *et al*, 2015; Mukherjee *et al*, 2016). In yeast, microautophagy has been involved in the degradation of multiple substrates, including peroxisomes (a process called "micropexophagy", historically the first form of yeast microautophagy to be described) (Farre & Subramani, 2004), portions of the nucleus (Kvam & Goldfarb, 2007), damaged mitochondria (Kissova *et al*, 2007), and lipid droplets (Vevea *et al*, 2015). In plants, microautophagy has been shown to mediate the degradation of anthocyanins (Chanoca *et al*, 2015). Finally, endosomal microautophagy degrades cytosolic proteins, either in bulk or selectively (only proteins containing a KFERQ-like motif recognized by HSPA8) (Sahu *et al*, 2011; Uytterhoeven *et al*, 2015; Mukherjee *et al*, 2016). Of note, some proteins internalized by multivesicular bodies through

direct membrane invagination can be spared from degradation and released in the extracellular microenvironment within exosomes (Record *et al*, 2014).

Arguably, microautophagy is the least studied form of autophagy, but a molecular signature of the process has begun to emerge. Thus, several forms of yeast microautophagy (e.g., micropexophagy) require some components of the macroautophagy machinery for cargo targeting and internalization, including (but perhaps not limited to) Atg7, Atg8, and Atg9 (Farre *et al*, 2008; Krick *et al*, 2008). Conversely, endosomal microautophagy relies on multiple endosomal sorting complexes required for transport (ESCRT) systems (Sahu *et al*, 2011; Liu *et al*, 2015b; Uytterhoeven *et al*, 2015; Mukherjee *et al*, 2016). In addition, the selective uptake of KFERQ-containing proteins by late endosomes in the course of endosomal microautophagy depends on HSPA8, reflecting its ability to directly interact with phosphatidylserine on (and hence deform) the outer endosomal membrane (Uytterhoeven *et al*, 2015; Morozova *et al*, 2016). Along similar lines, chaperone ATPase HSP104 (Hsp104) reportedly underlies microautophagic responses to lipid droplets in *Saccharomyces cerevisiae* (Vevea *et al*, 2015). However, the strict requirement of chaperones from the HSP70 protein family in other variants of microautophagy has not yet been documented. Of note, the yeast orthologue of mammalian NBR1, autophagy cargo receptor (NBR1; which is known to operate as a macroautophagy receptor, see below) reportedly underlies an ESCRT-dependent and ubiquitination-dependent microautophagic pathway in *Schizosaccharomyces pombe* (Liu *et al*, 2015b). It will be interesting to determine whether NBR1 and other components of this pathway also contribute to microautophagy in mammalian cells. Irrespectively, we propose to define *microautophagy* and *endosomal microautophagy* as types of autophagy in which the cargo is directly internalized in small vesicles that form at the surface of the lysosome/vacuole or late endosomes (multivesicular bodies), respectively, via ESCRT-independent (microautophagy) or ESCRT-dependent (endosomal microautophagy), mechanisms. In addition, selective endosomal microautophagy can be defined as an HSPA8-dependent autophagic response, but it can be differentiated from CMA based on (i) its dependence on ESCRT systems and (ii) its independence from a specific splicing variant of lysosomal-associated membrane protein 2 (LAMP2A, see below) (Table 1).

Chaperone-mediated autophagy

CMA involves the direct delivery of cytosolic proteins targeted for degradation to the lysosome (Kaushik & Cuervo, 2012). The distinctive feature of CMA is that neither vesicles nor membrane invaginations are required for substrate delivery to lysosomes, since substrates reach the lysosomal lumen through a protein-translocation complex at the lysosomal membrane (Kaushik & Cuervo, 2012). CMA only degrades soluble proteins bearing a KFERQ-like motif bound to HSPA8 (Dice, 1990), but not organelles, other macromolecules such as lipids, nucleic acids, or proteins integral to membranes (Chiang *et al*, 1989; Wing *et al*, 1991; Salvador *et al*, 2000). CMA has been shown to operate on a multitude of cytosolic proteins, hence exerting major regulatory functions in different pathophysiological scenarios such as metabolic regulation (Schneider *et al*, 2014; Kaushik & Cuervo, 2015), genome integrity preservation (Park *et al*, 2015), aging (Cuervo & Dice, 2000; Rodriguez-Muela *et al*, 2013; Schneider *et al*, 2015), T-cell activation (Valdor *et al*, 2014), neurodegeneration (Orenstein *et al*, 2013), and oncogenesis (Kon *et al*, 2011). Moreover, linear sequence analysis of the cytosolic proteome suggests that ~30% of its components may be degraded by CMA (Dice, 1990). Importantly, the translocation of CMA substrates across the lysosomal membrane relies on a dedicated molecular machinery that critically involves a specific splicing isoform of LAMP2, namely, LAMP2A (Cuervo & Dice, 1996). Thus, chaperone-bound autophagy substrates bind LAMP2A monomers on the cytosolic side of the lysosome, which stimulate the formation of an oligomeric LAMP2A translocation complex (Bandyopadhyay *et al*, 2008).

While unfolding and dissociating from chaperones (Salvador *et al*, 2000), CMA substrates are translocated into the lysosomal lumen through oligomeric LAMP2A complexes that are stabilized by a lysosomal pool of heat shock protein 90 alpha family class A member 1 (HSP90AA1; best known as HSP90) (Bandyopadhyay *et al*, 2008), and a cytosolic pool of glial fibrillary acidic protein (GFAP) (Bandyopadhyay *et al*, 2010). Lysosomal HSPA8 operates as an acceptor for CMA substrates, possibly by preventing cytosolic retrotranslocation (Agarraberes *et al*, 1997). Eventually, LAMP2A complexes are dismantled within lipid-rich microdomains of the lysosomal membrane by a mechanism that relies on HSPA8, followed by cathepsin A (CTSA)-catalyzed LAMP2A degradation (Kaushik *et al*, 2006). The CMA-supporting activity of GFAP is negatively regulated by phosphorylation, which is catalyzed by a pool of AKT serine/threonine kinase 1 (AKT1) that resides on the lysosomal surface (Arias *et al*, 2015). In this setting, dephosphorylation of AKT1 by PH domain and leucine-rich repeat protein phosphatase 1 (PHLPP1) counteracts the tonic activity of mechanistic target of rapamycin (MTOR) complex 2 (mTORC2), resulting in CMA activation (Arias *et al*, 2015). It remains to be determined to what extent CMA is conserved in lower organisms, since the splice variant of LAMP2 that is essential for CMA (i.e., LAMP2A) appeared relatively late in evolution (i.e., in birds) (Eskelinen *et al*, 2005). It has been suggested that selective endosomal microautophagy, which shares with CMA the dependence on KFERQ-like motives and HSPA8, constitutes an alternative to CMA in *D. melanogaster* (Mukherjee *et al*, 2016). Irrespective of this unknown, we propose to define *CMA* as an HSPA8-dependent autophagic response that relies on LAMP2A-mediated cargo translocation across the lysosomal membrane. In this context, it should be noted that other splicing isoforms of LAMP2 (including LAMP2B and LAMP2C) are dispensable for CMA but involved in macroautophagy (see below) (Eskelinen *et al*, 2005). This implies that genetic interventions aimed at specifically inhibiting CMA should not be directed to HSPA8 (which is also required for multiple forms of microautophagy), nor to *LAMP2* as a gene (Table 1).

Macroautophagy

Macroautophagy is the variant of autophagy best characterized thus far, at least in part owing to its easily distinguishable morphological features. Indeed, whereas microautophagy and CMA are not associated with major morphological changes in vesicular compartments, macroautophagic responses involve dedicated vesicles that can occupy (at a specific moment) a considerable part of the cytoplasm, an impressive phenomenon that attracted attention as early as in the late 1950s (Yang & Klionsky, 2010). These double-membraned vesicles, which are commonly known as autophagosomes, can

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Table 1. Main autophagy-related proteins in common model organisms.[a]

Homo sapiens	*Mus musculus*	*Drosophila melanogaster*	*Caenorhabditis elegans*	*Saccharomyces cerevisiae*
ACBD3 (PMP70)	ACBD3	Pmp70	PMP-2	–
ACBD5	ACBD5	–	–	Atg37
AMBRA1	AMBRA1	–	–	–
ATG2A, ATG2B	ATG2A, ATG2B	Atg2	ATG-2	Atg2
ATG3	ATG3	Atg3	ATG-3	Atg3
ATG4A, ATG4B, ATG4C, ATG4D	ATG4A, ATG4B, ATG4C, ATG4D	Atg4a, Atg4b	ATG-4.1, ATG-4.2	Atg4
ATG5	ATG5	Atg5	ATG-5	Atg5
ATG7	ATG7	Atg7	ATG-7	Atg7
ATG9A, ATG9B	ATG9A, ATG9B	Atg9	ATG-9	Atg9
ATG10	ATG10	Atg10	ATG-10	Atg10
ATG12	ATG12	Atg12	LGG-3	Atg12
ATG13	ATG13	Atg13	EPG-1 (ATG-13)	Atg13
ATG14 (ATG14L)	ATG14 (ATG14L)	Atg14	EPG-8	Atg14
ATG16L1	ATG16L1	Atg16	ATG-16.1, ATG-16.2	Atg16
ATG101	ATG101	Atg101	EPG-9	–
BCL2	BCL2	Debcl	CED-9	–
BCL2L13	BCL2L13	–	–	–
BECN1	BECN1	Atg6	BEC-1	Atg6
BNIP3 (NIP3)	BNIP3	–	DCT-1	–
BNIP3L (NIX)	BNIP3L	–	–	–
CALCOCO2 (NDP52)	CALCOCO2 (NDP52)	–	–	–
–	–	–	EPG-2	–
EI24 (EPG4)	EI24 (EPG4)	tank	EPG-4	–
EPG5	EPG5	Epg5	EPG-5	–
ENDOG	ENDOG	EndoG, Tengl1, Tengl2, Tengl3, Tengl4	CPS-6	Nuc1
FAM134B	FAM134B	–	–	Atg40
FANCC	FANCC	–	–	–
FUNDC1	FUNDC1	–	T06D8.7	–
GFAP	GFAP	–	–	–
HSP90AA1	HSP90AA1	Hsp83	DAF-21	Hsc82, Hsp82
HSPA8 (HSC70)	HSPA8 (HSC70)	Hsc70-1, Hsc70-2, Hsc70-3, Hsc70-4, Hsc70-5, Hsc70-6, Hsc70Cb	HSP-70	Ssa1, Ssa2, Ssa3, Ssa4
INPP5E	INPP5E	Inpp5e	–	–
LAMP1	LAMP1	Lamp1	LMP-1, LMP-2	–
LAMP2	LAMP2	–	–	–
LGALS3	LGALS3	–	–	–
LGALS8	LGALS8	–	–	–
MAP1LC3A, MAP1LC3B, MAP1LC3C, GABARAP, GABARAPL1, GABARAPL2	MAP1LC3A, MAP1LC3B, GABARAP, GABARAPL1, GABARAPL2[†]	Atg8a, Atg8b	LGG-1, LGG-2	Atg8
MTOR	MTOR	Tor	LET-363	Tor1
NBR1	NBR1	–	–	–
NRBF2	NRBF2	–	–	Atg38

[†]Correction added on 3 July 2017, after first online publication: the protein MAP1LC3C has been deleted.

Table 1 (continued)

Homo sapiens	*Mus musculus*	*Drosophila melanogaster*	*Caenorhabditis elegans*	*Saccharomyces cerevisiae*
OPTN	OPTN	–	–	–
PARK2	PARK2	park	PDR-1	–
PEX2	PEX2	Pex2	PRX-2	Pex2
PEX3	PEX3	Pex3	PRX-3	Pex3
PEX5	PEX5	Pex5	PRX-5	Pex5
PEX13	PEX13	Pex13	PRX-13	Pex13
PEX14	PEX14	Pex14	PRX-14	Pex14
PHB2	PHB2	Phb2	PHB-2	Phb2
PIK3C3 (VPS34)	PIK3C3 (VPS34)	Pi3K59F	VPS-34	Vps34
PIK3R4 (VPS15)	PIK3R4 (VPS15)	Vps15	VPS-15	Vps15
PINK1	PINK1	Pink1	PINK-1	–
PLEKHM1	PLEKHM1	CG6613	Y51H1A.2	–
PSMD4 (RPN10)	PSMD4 (RPN10)	Rpn10	RPN-10	Rpn10
RAB7A, RAB7B	RAB7A, RAB7B	Rab7	RAB-7	Ypt7
RAB11A	RAB11A	Rab11	RAB-11.1, RAB-11.2	Ypt31, Ypt32
RB1CC1 (FIP200)	RB1CC1	Atg17	EPG-7	Atg11, Atg17
RNF166	RNF166	–	–	–
RUBCN (RUBICON)	RUBCN (RUBICON)	CG12772	–	–
SMURF1	SMURF1	Smurf	–	–
SNX4	SNX4	–	SNX-3	Snx4 (Atg24)
SNX18	SNX18	Sh3px1	SNX-9	–
SQSTM1 (p62)	SQSTM1 (p62)	ref(2)P	SQST-1, SQST-2, SQST-3, SQST-4	–
STX17	STX17	Syx17	VF39H2L.1	–
TAX1BP1	TAX1BP1	–	–	–
TBK1	TBK1	LOC108141996	–	–
TECPR1	TECPR1	–	–	–
TFEB	TFEB	Mitf	HLH-30	–
TGM2	TGM2	Tg	–	–
TOLLIP	TOLLIP	–	TLI-1	Cue5
TRIM5	TRIM5	–	–	–
ULK1 (ATG1), ULK2	ULK1 (ATG1), ULK2	Atg1	UNC-51	Atg1
UVRAG	UVRAG	Uvrag	T23G11.7, Y34BA.2	Vps38
VCP	VCP	TER94	CDC-48.1, CDC-48.2	Cdc48
WDFY3 (ALFY)	WDFY3 (ALFY)	bchs	WDFY-3	–
WIPI1, WIPI2, WDR45B (WIPI3), WDR45 (WIPI4)	WIPI1, WIPI2, WDR45B (WIPI3), WDR45 (WIPI4)	Atg18a, Atg18b	ATG-18, EPG-6	Atg18, Atg21
VMP1	VMP1	Tango5	EPG-3	–
WAC	WAC	Wac	–	–
ZFYVE1 (DFCP1)	ZFYVE1 (DFCP1)	–	–	–

Yeast proteins with no known orthologues in *C. elegans*, *D. melanogaster*, *M. musculus* or *H. sapiens*: Atg19, Atg20, Atg23, Atg26, Atg27, Atg29, Atg30, Atg31, Atg32, Atg33, Atg34, Atg36, Atg39, Bre5, Doa1, Hsp104, Ubp3, Uth1.
[a]Excluding non-coding pseudogenes, as per https://www.ncbi.nlm.nih.gov/gene/; common aliases are indicated between brackets.

sequester large portions of the cytoplasm including entire organelles or parts thereof. This endows macroautophagy with a considerable catabolic potential that—in specific settings—can contribute to regulated cell death (RCD) (Galluzzi *et al*, 2016) or cellular atrophy leading to neurodegeneration (Cherra *et al*, 2010a,b; Zhu *et al*, 2013). The molecular machinery that executes and regulates

macroautophagy in organisms encompassing yeast, nematodes, flies, and mammals has been the subject of intense investigation throughout the past two decades (Noda & Inagaki, 2015; Antonioli *et al*, 2016). Although a detailed description of these pathways is not warranted here, a few functional modules of the macroautophagy apparatus are particularly important for this discussion. Indeed, the molecules that are part of these functional modules, their interactors and the processes they control have been extensively employed thus far to identify macroautophagic responses, though not always with precision. Efficient macroautophagic responses involving the formation of autophagosomes, their fusion with lysosomes, and lysosomal degradation have been associated with the activity of two ubiquitin-like conjugation systems (Noda & Inagaki, 2015; Antonioli *et al*, 2016). One relies on ATG7 and ATG10, which promote the conjugation of ATG5 to ATG12 in the context of a multiprotein complex containing autophagy-related 16-like 1 (ATG16L1) (Mizushima *et al*, 1998). Another one is mediated by ATG3 and ATG7, which together with the ATG12-ATG5:ATG16L1 complex conjugates phosphatidylethanolamine to microtubule-associated protein 1 light chain 3 beta (MAP1LC3B; best known as LC3B) and other orthologues of yeast Atg8 upon ATG4-dependent proteolytic maturation (Ichimura *et al*, 2000; Marino *et al*, 2010; Rockenfeller *et al*, 2015). Lipidated LC3 (often referred to as LC3-II) is generated onto forming autophagosomes and allows for substrate uptake upon binding to several autophagy receptors (Kabeya *et al*, 2000; Stolz *et al*, 2014; Wild *et al*, 2014). Importantly, robust data suggest that the ATG conjugation systems and Atg8-like proteins are not strictly required for the formation of autophagosomes, as classically thought (although their absence greatly reduces the efficiency of the process), but also contribute to autophagosome extension around large substrates and closure, the fusion of autophagosomes with lysosomes, and the degradation of the inner autophagosomal membrane (Nguyen *et al*, 2016; Tsuboyama *et al*, 2016).

In response to commonly studied stimuli including starvation, autophagosome formation is initiated by the assembly and activation of a multiprotein complex containing ATG13, ATG101, RB1 inducible coiled-coil 1 (RB1CC1; best known as FIP200) and *unc-51*-like autophagy activating kinase 1 (ULK1, the mammalian orthologue of yeast Atg1) at ATG9-containing membranes, followed by ULK1-dependent ATG9 phosphorylation (Orsi *et al*, 2012; Papinski *et al*, 2014; Stanley *et al*, 2014; Joachim *et al*, 2015; Karanasios *et al*, 2016). This event initiates the elongation of pre-autophagosomal membranes upon incorporation of phospholipids from various sources including the endoplasmic reticulum (ER), recycling endosomes, and mitochondria (Lamb *et al*, 2013), and allows for the recruitment of a multiprotein complex with Class III phosphatidylinositol 3-kinase (PI3K) activity, which contains beclin 1 (BECN1), phosphatidylinositol 3-kinase catalytic subunit type 3 (PIK3C3; best known as VPS34), phosphoinositide-3-kinase regulatory subunit 4 (PI3KR4; best known as VPS15) (Kihara *et al*, 2001a, b), the sensor of membrane curvature ATG14 (also known as ATG14L or BARKOR) (Itakura *et al*, 2008; Sun *et al*, 2008; Matsunaga *et al*, 2009; Zhong *et al*, 2009; Fan *et al*, 2011), and nuclear receptor binding factor 2 (NRBF2) (Lu *et al*, 2014a). On activation, VPS34 produces phosphatidylinositol 3-phosphate (PI3P), which further supports the expansion of autophagosomal membranes until closure by engaging PI3P-binding ATG proteins and members of the WIPI family (Proikas-Cezanne *et al*, 2015). Both the ULK1 and autophagy-specific Class III PI3K complexes are highly regulated. One of the main regulators of macroautophagy is MTOR complex 1 (mTORC1), which robustly suppresses autophagosome formation by catalyzing the inactivating phosphorylation of ATG13 and ULK1 (Jung *et al*, 2009; Nicklin *et al*, 2009; Nazio *et al*, 2013) Moreover, mTORC1 inhibits macroautophagic responses by preventing the nuclear translocation of transcription factor EB (TFEB, a master transcriptional regulator of lysosomal biogenesis and macroautophagy) upon phosphorylation on S142 (Settembre *et al*, 2011, 2012). Such a multipronged inhibitory network is disrupted upon mTORC1 inactivation by AMP-activated protein kinase (AMPK), which responds to reduced ATP levels and consequent AMP accumulation (Inoki *et al*, 2002). AMPK also catalyzes activating phosphorylation events on ULK1 (Lee *et al*, 2010; Egan *et al*, 2011; Kim *et al*, 2011) and BECN1 (Kim *et al*, 2013b). In mammalian cells, ULK1 directly phosphorylates BECN1, resembling AMPK in its VPS34-stimulatory effects (Russell *et al*, 2013), and ATG14 (Park *et al*, 2016; Wold *et al*, 2016). The autophagy-specific Class III PI3K complex is regulated by several interactors, including the VPS34 activator autophagy and beclin 1 regulator 1 (AMBRA1, originally "activating molecule in Beclin 1-regulated autophagy"), as well as the BECN1 inhibitor BCL2, which also interacts with ATG12 (Liang *et al*, 1999; Pattingre *et al*, 2005; Fimia *et al*, 2007; Zalckvar *et al*, 2009; Rubinstein *et al*, 2011).

Once autophagosomes have enclosed autophagy substrates, they can fuse with late endosomes or lysosomes to form amphisomes or autolysosomes (see below for definitions). The molecular machinery that is responsible for these fusion events involve dozens of proteins, most of which are shared with the endocytic pathway (Amaya *et al*, 2015; Antonioli *et al*, 2016). In this setting, an important role is mediated by the activation of the GTPase RAB7A, member RAS oncogene family (RAB7A), which is required for autophagosome maturation (Gutierrez *et al*, 2004; Jager *et al*, 2004; Liang *et al*, 2008), the RAB7 effector pleckstrin homology and RUN domain containing M1 (PLEKHM1) (McEwan *et al*, 2015), the PI3P-binding protein tectonin beta-propeller repeat containing 1 (TECPR1) (Chen *et al*, 2012), ectopic P-granules autophagy protein 5 homolog (EPG5) (Tian *et al*, 2010), inositol polyphosphate-5-phosphatase E (INPP5E) (Hasegawa *et al*, 2016), syntaxin 17 (STX17), and other soluble N-ethylmaleimide-sensitive factor activating protein receptor (SNARE) proteins (Fader *et al*, 2009; Nair *et al*, 2011; Itakura *et al*, 2012), as well as homotypic fusion and vacuole protein sorting (HOPS) complexes (McEwan *et al*, 2015). ATG14, LAMP2B (but not LAMP2A) as well as phosphorylated and lipidated LC3 are also involved in the formation of autolysosomes (Eskelinen *et al*, 2005; Diao *et al*, 2015; Wilkinson *et al*, 2015; Nguyen *et al*, 2016). Conversely, RUN and cysteine-rich domain containing beclin 1 interacting protein (RUBCN; best known as RUBICON) negatively regulates the fusion of autophagosomes with lysosomes upon interacting with VPS34 (Matsunaga *et al*, 2009). Degradation of autophagy substrates proceeds as the lysosomal lumen is acidified (owing to the activity of an ATP-dependent proton pump commonly known as V-type ATPase) (Mindell, 2012), upon disassembly of the inner autophagosomal membrane supported by the ATG conjugation systems (Tsuboyama *et al*, 2016). Finally, mTORC1 reactivation inhibits macroautophagy as it promotes so-called autophagic lysosome reformation (ALR), a

process whereby proto-lysosomal vesicles extruding from autolysosomes mature to regenerate the lysosomal compartment (Yu *et al*, 2010).

Several of the proteins mentioned above including ATG3, ATG5, ATG7, ATG9, ATG13, ATG16L1, ULK1, BECN1, and VPS34 have been considered as strictly required for macroautophagic responses (irrespective of their functions in autophagy-independent processes) (Codogno *et al*, 2012). At least in part, such a view originated from the embryonic or post-natal lethality caused in mice by the genetic ablation of any of these components of the macroautophagy machinery at the whole-body level (Qu *et al*, 2003; Yue *et al*, 2003; Kuma *et al*, 2004; Komatsu *et al*, 2005; Gan *et al*, 2006; Saitoh *et al*, 2008, 2009; Sou *et al*, 2008), which is likely to reflect the key role of macroautophagy in development and adult tissue homeostasis (although such a general phenotype might also stem from autophagy-independent functions of these proteins). In addition, both pharmacological and genetic interventions targeting these and other components of the macroautophagy apparatus have been associated with autophagic defects in hundreds of experimental settings, *in vitro* and *in vivo*. However, the discovery of *bona fide* macroautophagic responses occurring independently of ATG3, ATG5, ATG7, ULK1, BECN1, VPS34, and its product (PI3P) (Zhu *et al*, 2007; Nishida *et al*, 2009; Chang *et al*, 2013; Niso-Santano *et al*, 2015; Vicinanza *et al*, 2015) casted doubts on the exclusive requirement of these factors for all forms of macroautophagy (Klionsky *et al*, 2016). The existence of ATG3-, ATG5-, ATG7-, ULK1-, BECN1-, VPS34-, and PI3P-independent forms of macroautophagy lent further support to the hypothesis that the molecular mechanisms underlying macroautophagic responses exhibit considerable degree of redundancy (at least in mammals) (Nishida *et al*, 2009; Chu, 2011; Chang *et al*, 2013; Niso-Santano *et al*, 2015; Vicinanza *et al*, 2015). This notion had previously been postulated based on the observation that some components of the macroautophagy apparatus have multiple functional homologues. For instance, the human genome codes for at least six distinct Atg8-like proteins, namely microtubule-associated protein 1 light chain 3 alpha (MAP1LC3A; best known as LC3A), LC3B, microtubule-associated protein 1 light chain 3 gamma (MAP1LC3C; best known as LC3C), GABA type A receptor-associated protein (GABARAP), GABA type A receptor-associated protein-like 1 (GABARAPL1), and GABA type A receptor-associated protein-like 2 (GABARAPL2; best known as GATE-16) (Shpilka *et al*, 2011) (Table 1).

Throughout the past decade, the terms "canonical" and "non-canonical" have been extensively employed to (i) refer to non-degradative functions of macroautophagy (e.g., unconventional secretion) (Ponpuak *et al*, 2015), or (ii) discriminate between those macroautophagic responses that critically rely on ATG3-, ATG5-, ATG7-, ULK1-, BECN1-, and VPS34-mediated PI3P production and those that do not (Codogno *et al*, 2012; Ktistakis & Tooze, 2016). Although this latter use of the adjectives "canonical" and "non-canonical" may be advantageous as it refers to molecular signatures that are shared by various instances of macroautophagy, we fear that it might be rather misleading, for at least two reasons. First, they implicitly convey the notion that some macroautophagic responses are frequent and observable in many distinct experimental settings, while others are relatively exceptional. The literature describes hundreds of scenarios in which macroautophagy can be slowed down by the inhibition of ATG3-, ATG5-, ATG7-, ULK1-, BECN1-, and VPS34-dependent PI3P production, but only a few instances of ATG3-, ATG5-, ATG7-, ULK1-, BECN1-, VPS34,- and PI3P-independent macroautophagic responses (Nishida *et al*, 2009; Niso-Santano *et al*, 2015; Vicinanza *et al*, 2015). However, this imbalance might stem from an observational bias linked to the stimuli used to elicit autophagy (starvation, rapamycin or targeted cellular damage) and/or to the biomarkers used so far to monitor macroautophagic responses (such as LC3 lipidation) (Klionsky *et al*, 2016). Second, and perhaps most important, a real consensus on the set of features that would characterize "canonical" versus "non-canonical" macroautophagy has never been reached. Thus, while some authors have used the term "non-canonical" for ATG5-dependent, BECN1-independent cases of macroautophagy (Niso-Santano *et al*, 2015; Huang & Liu, 2016), others have employed the same expression for ULK1-independent, ATG5- and BECN1-dependent macroautophagic responses (Martinez *et al*, 2016). To avoid confusion, we propose to avoid terms such as "*canonical*" and "*non-canonical*". Rather, we encourage the use of explicit expressions such as "ATG5-dependent", "BECN1-independent" and alike, provided that such a dependence/independence has been experimentally verified. Of note, this recommendation does not intend to imply the existence of distinct pathways that fully depend or not on specific components of the macroautophagy apparatus, but to support the description of a specific instance of macroautophagy based on experimental validation.

As for the definition of *macroautophagic responses*, relying upon specific components of the underlying molecular apparatus may also be relatively misleading. We propose therefore a functional definition of macroautophagy as a type of autophagic response (i.e., a response that involves the lysosomal degradation of a cytosolic entity, see above) that relies on autophagosomes, which can be subtyped based upon dependence on specific proteins. Comprehensive guidelines provide robust methods to monitor the formation of functional autophagosomes and autophagic flux (Klionsky *et al*, 2016). We surmise that a common molecular signature of macroautophagic responses may be difficult to identify, at least in part owing to the high degree of redundancy and interconnectivity of the process (at least in mammalian cells).

Non-selective and selective types of autophagy

Micro- and macroautophagic responses can involve disposable cytoplasmic components in a relatively non-selective manner. Upon lysosomal degradation, these autophagy substrates fuel bioenergetic metabolism or repair processes (Liu *et al*, 2015a; Sica *et al*, 2015). In addition, microautophagy, macroautophagy, and CMA can operate in a specific manner, through a mechanism that involves the recognition of autophagy substrates by dedicated receptors (Farre & Subramani, 2016). In this setting, it is useful to remember that the specificity of autophagic responses is highly affected by the mechanisms of substrate delivery to lysosomes. Thus, whereas CMA appears as a highly selective type of autophagy (as it virtually operates only on cytosolic proteins containing KFERQ-like motives bound to HSPA8 and compatible with LAMP2A-mediated translocation), both microautophagy and macroautophagy can exhibit incomplete specificity under specific conditions (reflecting the relatively "leaky" processes of lysosomal invagination and autophagosome formation, respectively) (Sica *et al*, 2015; Zaffagnini & Martens, 2016). This notion should be kept under attentive consideration

when specific instances of autophagy (see below) are measured. The literature offers a collection of articles in which specificity was not addressed, as investigators focused on the degradation of a single substrate (e.g., damaged mitochondria) but did not monitor to which extent other cytoplasmic entities were also degraded. Thus, it may be difficult to differentiate between non-selective micro- or macroautophagic responses and their specific counterparts, especially for some substrates like mitochondria. Indeed, mitophagy (see below for a definition) is arguably the best-characterized form of selective macroautophagy (at least in mammalian cells), but parts of the mitochondrial network are also degraded in the course of macroautophagic responses driven by bioenergetic needs (Gomes *et al*, 2011a,b). We propose to define specific instances of micro- and macroautophagy based on the enrichment of a precise autophagy substrate, coupled to requirement of specific molecular factors (such as autophagy receptors), which may be used to selectively monitor or experimentally manipulate the process (Table 1).

Mitophagy Mitophagy can be defined as the specific removal of damaged or excess mitochondria by micro- or macroautophagy. Microautophagic responses preferentially targeting mitochondria have been observed in yeast cells submitted to nitrogen starvation (Kissova *et al*, 2007). In this system, the microautophagic response depends on SUN family protein UTH1 (Uth1), an integral factor of the inner mitochondrial membrane (Kissova *et al*, 2007). Whether Uth1 is the actual receptor for mitochondrial microautophagy, however, remains to be determined. Conversely, macroautophagic responses specific for mitochondria have been described in a wide panel of model organisms, including yeast, nematodes, flies, and mammals. This process contributes to the removal of superfluous mitochondria that have no functional defects *a priori*, as well as to the degradation of mitochondria that are damaged beyond repair, hence dysfunctional and potentially cytotoxic (which is critical for the maintenance of cellular homeostasis, especially in highly metabolic tissues such as the brain) (Palikaras & Tavernarakis, 2014). Two physiological settings exemplify the macroautophagic removal of functional mitochondria: (i) the maturation of reticulocytes and consequent formation of mature erythrocytes, a setting in which mitophagy critically relies on BCL2 interacting protein 3-like (BNIP3L; best known as NIX) and the complete removal of mitochondria may also depend on unconventional secretion (Sandoval *et al*, 2008; Mortensen *et al*, 2010; Novak *et al*, 2010; Griffiths *et al*, 2012; Fader *et al*, 2016); (ii) the first steps of embryonic development (Al Rawi *et al*, 2011; Sato & Sato, 2011), in which paternal mitochondria undergo fission, mitochondrial 1 (FIS1)-dependent fragmentation (Rojansky *et al*, 2016; Wang *et al*, 2016), lose transmembrane potential (Rojansky *et al*, 2016; Wang *et al*, 2016) and are removed by a mitophagic response depending on endonuclease G (ENDOG; at least in *Caenorhabditis elegans*) (Zhou *et al*, 2016), prohibitin 2 (PHB2) (Wei *et al*, 2017), PTEN-induced putative kinase 1 (PINK1), and Parkinson disease (autosomal recessive, juvenile) 2, parkin (PARK2) (in mammals, but not in *D. melanogaster*) (Politi *et al*, 2014; Rojansky *et al*, 2016). In this scenario, CPS-6 (the worm orthologue of ENDOG) promotes mitophagy via a poorly characterized mechanism that involves the degradation of the mitochondrial genome (Zhou *et al*, 2016), whereas PHB2 and the PINK1-PARK2 system contribute to the generation of tags recognizable by LC3 or autophagy receptors, respectively (Geisler *et al*, 2010; Narendra *et al*, 2010; Wei *et al*, 2017).

The selective removal of depolarized mitochondria also involves the PINK1-PARK2 system and PHB2 (Clark *et al*, 2006; Park *et al*, 2006), which generate ubiquitin and non-ubiquitin tags at damaged mitochondrial membranes to allow recognition by sequestosome 1 (SQSTM1, best known as p62) (to a limited extent), optineurin (OPTN), calcium binding and coiled-coil domain 2 (CALCOCO2; best known as NDP52), and LC3 (Wong & Holzbaur, 2014; Heo *et al*, 2015; Lazarou *et al*, 2015; Moore & Holzbaur, 2016; Wei *et al*, 2017). Cardiolipin, a mitochondrial lipid, has also been proposed to directly interact with LC3 upon mitochondrial damage caused by a variety of stimuli (Chu *et al*, 2013; Kagan *et al*, 2016). FUN14 domain containing 1 (FUNDC1), a protein of the outer mitochondrial membrane, operates as autophagy receptor in response to hypoxia (Liu *et al*, 2012). Finally, SMAD-specific E3 ubiquitin-protein ligase 1 (SMURF1), peroxisomal biogenesis factor 3 (PEX3), PEX13, various members of the Fanconi anemia (FA) protein family and transglutaminase 2 (TGM2) have also been involved in the regulation or execution of mitophagy, although their exact role remains to be elucidated (Orvedahl *et al*, 2011; Rossin *et al*, 2015; Lee *et al*, 2017; Sumpter *et al*, 2016). Atg32 is the main receptor for macroautophagic responses targeting dispensable mitochondria in yeast (Kanki *et al*, 2009; Okamoto *et al*, 2009), and BCL2-like 13 (BCL2L13) has been suggested to play analogous functions in mitophagy in mouse and human cells (Murakawa *et al*, 2015). In *C. elegans*, macroautophagic responses specific for mitochondria are coordinated with mitochondrial biogenesis owing to the coordinated activity of the BNIP3 homologue DCT-1 and the transcription factor SNK-1 (Palikaras *et al*, 2015).

Pexophagy Pexophagy is a macroautophagic response preferentially targeting peroxisomes. In yeast, a large supramolecular complex is responsible for the selective recognition of peroxisomes by the molecular machinery for macroautophagy and their actin-dependent transport to the vacuole (Reggiori *et al*, 2005). This complex includes the peroxisomal proteins Pex3 (Burnett *et al*, 2015), Pex14 (Zutphen *et al*, 2008) as well as Atg37 (Nazarko *et al*, 2014), which are bound by Atg30 (Burnett *et al*, 2015), Atg11 (Burnett *et al*, 2015; Torggler *et al*, 2016), and Atg36 (Motley *et al*, 2012; Tanaka *et al*, 2014). In mammalian cells, pexophagy proceeds upon the PEX2- and PEX3-dependent ubiquitination of multiple peroxisomal proteins including PEX5 and ATP-binding cassette subfamily D member 3 (ABCD3; best known as PMP70), which are recognized by the autophagy receptors p62 and NBR1 (Deosaran *et al*, 2013; Yamashita *et al*, 2014; Sargent *et al*, 2016). Mammalian pexophagy is highly responsive to oxidative stress, possibly as a consequence of cytoplasmic ATM activation or endothelial PAS domain protein 1 (EPAS1; best known as HIF-2α) signaling (Walter *et al*, 2014; Zhang *et al*, 2015). Of note, the selective degradation of peroxisomes in yeast has also been shown to occur through a selective form of microautophagy termed micropexophagy (Farre & Subramani, 2004).

Nucleophagy Nucleophagy can be defined as an autophagic response selectively targeting portions of the nucleus. In yeast, two

distinct forms of nucleophagy have been described: (i) a microautophagic form that relies on the autophagy receptor Nvj1, the vacuolar protein Vac8 and members of the oxysterol-binding protein (OSBP) family (Roberts *et al*, 2003; Kvam & Goldfarb, 2004), which has been dubbed "piecemeal microautophagy of the nucleus"; and (ii) a variant that does not require Nvj1, Vac8 but does involve components of the macroautophagy machinery, such as Atg3 and Atg4 (but not Atg6, the yeast orthologue of BECN1) (Krick *et al*, 2008; Mijaljica *et al*, 2012), and the autophagy receptor Atg39 (Mochida *et al*, 2015). Nucleophagy also occurs in mammalian cells (Park *et al*, 2009), in which it contributes to the maintenance of genomic integrity (Rello-Varona *et al*, 2012; Dou *et al*, 2015). Lamin B1 (LMNB1) has been identified as the nuclear protein responsible for a variant of nucleophagy in mammalian cells (Dou *et al*, 2015).

Reticulophagy Reticulophagy is the preferential autophagic degradation of portions of the ER. According to some authors, reticulophagy (also called
ER-phagy) occurs independently of both the micro- and macroautophagy machinery, at least in yeast (Schuck *et al*, 2014), but is regulated by the Rab family GTPase Ypt1 (Lipatova *et al*, 2013). Other authors, however, provided evidence suggesting that reticulophagy constitutes a specific form of macroautophagy that relies on the autophagy receptors Atg39 and Atg40 (in yeast), or their mammalian orthologue family with sequence similarity 134 member B (FAM134B) (in human and mouse cells) (Khaminets *et al*, 2015; Mochida *et al*, 2015). In *S. cerevisiae*, reticulophagy also involves Atg11 (Mochida *et al*, 2015) and Sec63 complex subunit SEC62 (Sec62) (Fumagalli *et al*, 2016).

Ribophagy Ribophagy is a specific autophagic response targeting ribosomes. In yeast, ribophagy involves ribosomal de-ubiquitination by the mRNA-binding ubiquitin-specific protease Ubp3 and its cofactors Bre5, Doa1 (also known as Ufd3), and Cdc48 (Kraft *et al*, 2008; Ossareh-Nazari *et al*, 2010) and requires Atg11 (Waliullah *et al*, 2017). Conversely, the autophagic removal of dispensable ribosomes is negatively regulated by listerin E3 ubiquitin-protein ligase 1 (Ltn1)-dependent ubiquitination (Ossareh-Nazari *et al*, 2014), and possibly by NEDD4 family E3 ubiquitin-protein ligase Rsp5 (Shcherbik & Pestov, 2011). Ubp3 has also been involved in the autophagic and proteasomal removal of translation and RNA turnover factors during nitrogen starvation (Kelly & Bedwell, 2015). Ribophagy driven by nutrient starvation in yeast is accompanied by bulk RNA degradation within the vacuole (Huang *et al*, 2015). Interestingly, some plants exhibit a microautophagic variant of ribophagy (Niki *et al*, 2014). To the best of our knowledge, ribophagic responses in mammalian cells have not yet been described.

Aggrephagy Aggrephagy can be defined as an autophagic response specific for protein aggregates. Aggrephagy has been described in a variety of model organisms, including yeast (Lu *et al*, 2014b), worms (Jia *et al*, 2007; Lu *et al*, 2013), flies (Simonsen *et al*, 2008), plants (Toyooka *et al*, 2006), and mammals (Bjorkoy *et al*, 2005; Hara *et al*, 2006; Komatsu *et al*, 2006). The macroautophagic disposal of protein aggregates is particularly relevant for the preservation of cellular homeostasis, especially in the context of neurodegenerative disorders (Menzies *et al*, 2015). Besides relying on the macroautophagy machinery and often on substrate ubiquitination, mammalian aggrephagy involves the autophagy receptors p62 (which can form insoluble aggregates itself) (Bjorkoy *et al*, 2005; Komatsu *et al*, 2007; Pankiv *et al*, 2007; Kirkin *et al*, 2009b), NBR1 (an orthologue of which participates in plant aggrephagy) (Kirkin *et al*, 2009a,b), OPTN (Korac *et al*, 2013), and toll-interacting protein (TOLLIP) (Lu *et al*, 2014b), as well as the p62-binding proteins WD repeat and FYVE domain containing 3 (WDFY3; best known as ALFY) (Simonsen *et al*, 2004; Filimonenko *et al*, 2010) and TGM2 (D'Eletto *et al*, 2012). However, it is worth noting that the redundancy between these factors and their specific roles in the degradation of different substrates has not been extensively explored. In yeast, the ubiquitin-binding protein Cue5 (the orthologue of mammalian TOLLIP) operates as autophagy receptor for aggrephagic responses (Lu *et al*, 2014b). In *D. melanogaster* the control of proteostasis by aggrephagy impinges on forkhead box, subgroup O (FOXO)-dependent transcription (Demontis & Perrimon, 2010). Importantly, LC3 can accumulate at protein aggregates in a p62-dependent but autophagosome-independent manner (Kuma *et al*, 2007; Shvets & Elazar, 2008). This adds to the potential sources of bias deriving from the use of GFP-LC3 aggregation as a standalone biomarker for macroautophagy (see above). HSPA8 as well as other chaperones and co-chaperones have been involved in a specific form of aggrephagy commonly known as "chaperone-assisted selective autophagy" (CASA) (Arndt *et al*, 2010). CASA differs from endosomal microautophagy and CMA in its dependence on multiple components of the macroautophagy apparatus, *de facto* constituting a selective form of macroautophagy (Arndt *et al*, 2010).

Lipophagy Lipophagy is the selective autophagic degradation of neutral lipid droplets. Originally discovered in the mammalian system, where it involves the molecular machinery for macroautophagy (Singh *et al*, 2009), lipophagy also occurs in worms and in yeast. In *C. elegans*, lipophagy involves lysosomal lipases such as LIPL-4, which play key signaling roles in longevity (Lapierre *et al*, 2011; O'Rourke & Ruvkun, 2013; Folick *et al*, 2015). In yeast, it involves a microautophagic process (Wang *et al*, 2014; Vevea *et al*, 2015). However, there are contradicting reports on the molecular requirements for *S. cerevisiae* lipophagic responses to intracellular lipid accumulation (Wang *et al*, 2014; Vevea *et al*, 2015). Thus, while some authors propose that lipophagy in yeast does not involve Atg7 but requires ESCRT components (Vevea *et al*, 2015), other authors favor the interpretation that lipophagic responses in yeast depend on Atg7 and several other components of the macroautophagy machinery (even though they manifest with a microautophagic appearance and proceed in the absence of autophagosomes) (Wang *et al*, 2014). In mammalian cells, lipophagy is coordinated by transcriptional programs depending on nuclear receptor subfamily 1 group H member 4 (NR1H4; best known as FXR), cAMP responsive element binding protein 1 (CREB), and peroxisome proliferator-activated receptor alpha (PPARA) (Lee *et al*, 2014; Seok *et al*, 2014). Interestingly, the CMA-dependent degradation of lipid droplet-associated proteins such as perilipin 2 (PLIN2) and PLIN3 precedes and facilitates lipolysis (Kaushik & Cuervo, 2015, 2016), demonstrating the existence of intimate cross talk between different forms of autophagy in the control of intracellular homeostasis. Moreover, several autophagy genes including *bec-1* (the worm orthologue of *BECN1*) are required for the accumulation of neutral lipids in the intestine of developing

C. elegans (Lapierre *et al*, 2013), pointing to a broader implication of autophagy in systemic lipid homeostasis.

Bacterial xenophagy Bacterial xenophagy is the macroautophagic removal of cytoplasmic bacteria, that is, bacteria that escape the phagosomal compartment upon phagocytosis, and damaged bacteria-containing phagosomes. As mentioned above, bacterial xenophagy must be conceptually discriminated from efficient phagocytosis, a setting in which bacteria never gain direct access to the cytosolic milieu (Huang & Brumell, 2014). Xenophagic responses targeting bacteria constitute a first, cell-autonomous line of innate defense against prokaryotic infections (Deretic *et al*, 2013). Accordingly, multiple bacteria have evolved strategies to actively inhibit autophagic responses in the host (Galluzzi *et al*, 2017b). In mammalian cells, cytoplasmic bacteria are rapidly recognized by various autophagy receptors including p62, OPTN, NDP52, and Tax1-binding protein 1 (TAX1BP1), via a mechanism that relies on receptor phosphorylation by TANK1-binding kinase 1 (TBK1) (Thurston *et al*, 2009; Wild *et al*, 2011; Tumbarello *et al*, 2015) and ubiquitination by ring finger protein 166 (RNF166) (Heath *et al*, 2016). Additional proteins that direct the formation and expansion of autophagosomes to sites of bacterial invasions include (but may not be limited to) WD repeat domain, phosphoinositide interacting 2 (WIPI2), and its interactor TECPR1, which are recruited in a TBK1-dependent manner (Ogawa *et al*, 2011; Thurston *et al*, 2016), as well as the pattern recognition receptors nucleotide-binding oligomerization domain containing 1 (NOD1) and NOD2, which physically interact with ATG16L1 and immunity-related GTPase M (IRGM) upon recognition of bacterial muramyl dipeptide (Cooney *et al*, 2010; Travassos *et al*, 2010; Chauhan *et al*, 2015). Besides operating as a receptor for the recruitment of forming autophagosomes to invading bacteria, NDP52 supports autophagosome maturation upon interaction with LC3A, LC3B, LC3C, GABARAPL2, and myosin VI (MYO6) (von Muhlinen *et al*, 2012; Verlhac *et al*, 2015). Ubiquitin D (UBD; best known as FAT10) has also been involved in the rapid and transient recognition of phagosome-escaping bacteria, and FAT10 deficiency has been associated with increased susceptibility to *Salmonella typhimurium* infection in mice (Spinnenhirn *et al*, 2014). The molecular mechanisms through which FAT10 supports xenophagy, however, remain to be clarified. Interestingly, xenophagic responses targeting damaged phagosomes and their bacterial cargo have been described. This particular variant of xenophagy relies on galectin 8 (LGALS8) or galectin 3 (LGALS3), both of which tag damaged endosomes (Chauhan *et al*, 2016), as well as on NDP52 (Thurston *et al*, 2012; Kim *et al*, 2013a; Li *et al*, 2013) and/or various members of the TRIM protein family as receptors or receptor regulators (see below for a definition) (Kimura *et al*, 2015, 2016). Although
xenophagic responses have mainly been studied in the mammalian system, there are *bona fide* instances of xenophagy in *D. melanogaster*, in which it also operates at the boundary of innate pattern recognition (Wu *et al*, 2007; Yano *et al*, 2008; Kim *et al*, 2012), *C. elegans* (Jia *et al*, 2009; Zou *et al*, 2014) and *Dictyostelium discoideum* (Jia *et al*, 2009).

Viral xenophagy Viral xenophagy (virophagy) is a macroautophagic response targeting fully formed cytoplasmic virions or components thereof. The first description of endogenous membranes engulfing cytoplasmic viruses dates back to the late 1990s (Schlegel *et al*, 1996), and it is now clear that virophagy occupies a position similar to that of bacterial xenophagy in the first line of defense against pathogens (Paul & Munz, 2016). In line with this notion, several defects in the molecular machinery for macroautophagy—such as the genetic inhibition of *Atg5* in mice—render animals more susceptible to succumb to infection (Orvedahl *et al*, 2010). This holds true not only in mammalian systems, but also in plants (Liu *et al*, 2005), flies (Nakamoto *et al*, 2012; Moy *et al*, 2014) and perhaps nematodes (Bakowski *et al*, 2014). Moreover, HIV-1^+ patients who remain clinically stable for years in the absence of therapy (so-called long-term non-progressors) display high baseline levels of autophagy in peripheral blood mononuclear cells (Nardacci *et al*, 2014). Accordingly, multiple viruses have evolved strategies to avoid host virophagic responses, including the expression of BECN1 inhibitors (Orvedahl *et al*, 2007; Levine *et al*, 2011) or proteins that inhibit the autophagosomal-lysosomal fusion (Gannage *et al*, 2009). Besides relying on the core macroautophagy machinery, efficient virophagic responses involve p62 and tripartite motif containing 5 (TRIM5) as receptors (Orvedahl *et al*, 2010; Mandell *et al*, 2014), proteins that participate in mitophagy, such as SMURF1 (Orvedahl *et al*, 2011), Fanconi anemia complementation group C (FANCC) (Sumpter *et al*, 2016), and PEX13 (Lee *et al*, 2017), as well as the phosphorylation of eukaryotic translation initiation factor 2A (EIF2A) (Talloczy *et al*, 2002).

Proteaphagy Proteaphagy is a term coined to indicate macroautophagic responses specific for inactive proteasomes. In *Arabidopsis thaliana*, proteaphagy relies on the proteasomal component regulatory particle non-ATPase 10 (RPN10), which operates as a *bona fide* autophagy receptor to bridge ubiquitinated proteasome subunits to ATG8 (Marshall *et al*, 2015). In yeast, Rpn10 is dispensable for proteaphagy (Waite *et al*, 2016) but a similar function is mediated by Cue5 (Marshall *et al*, 2016), drawing an interesting parallelism with aggrephagy (see above). Besides involving Atg7, optimal proteaphagic responses in *S. cerevisiae* rely on the co-chaperone Hsp42 (Marshall *et al*, 2016). Thus, it is tempting to speculate that the macroautophagic disposal of inactive proteasomes may proceed upon their accumulation in aggregates, at least in yeast. Mammalian cells subjected to starvation and other stressful conditions mount proteaphagic responses that mainly on p62 as a receptor (Cuervo *et al*, 1995; Cohen-Kaplan *et al*, 2016).

Lysophagy Lysophagy is the specific macroautophagic disposal of damaged lysosomes in mammalian cells. Several lysosomotropic agents as well as monosodium urate (MSU) and silica have been shown to promote lysosomal damage followed by ubiquitination and recruitment of the macroautophagy machinery (Hung *et al*, 2013; Maejima *et al*, 2013), a process that may be directed by the common marker of endovesicular damage LGALS3 (Kawabata & Yoshimori, 2016). Most of the molecular details underlying lysophagy, however, remain to be determined. Similarly, if and how a lysophagy-like mechanism contributes to the preservation of vacuolar homeostasis in yeast and plants remains obscure.

Other specific forms of autophagy Additional instances of selective macroautophagy have been described, mostly based on cargo selectivity. These include (but are likely not limited to): *myelinophagy*

(targeting myelin in Schwann cells) (Gomez-Sanchez *et al*, 2015), *zymophagy* (targeting zymogen granules in pancreatic acinar cells) (Grasso *et al*, 2011), *granulophagy* (targeting stress granules) (Buchan *et al*, 2013), and *ferritinophagy* (targeting ferritin via the receptor nuclear receptor coactivator 4, NCOA4) (Dowdle *et al*, 2014; Mancias *et al*, 2014). Finally, macroautophagy has been involved in the degradation of specific proteins owing to their ability to physically interact with members of the Atg8 protein family. This applies, for instance, to the centriole and centriolar satellite protein OFD1, whose degradation by macroautophagy has a major impact on the regulation of ciliogenesis (Tang *et al*, 2013). A term to indicate such a protein-specific variant of macroautophagy has yet to be proposed.

Autophagic flux

All forms of autophagy are multistep processes during which autophagy substrates are recognized, isolated (biochemically and/or physically) from the cytoplasmic milieu, and delivered to lysosomes for degradation. In physiological conditions, microautophagy, CMA, and macroautophagy proceed at baseline levels, hence contributing to the preservation of cellular homeostasis as they avoid the accumulation of potentially cytotoxic entities that may accumulate as a result of normal cellular functions (e.g., damaged mitochondria) (Li *et al*, 2012; Cuervo & Wong, 2014; Sica *et al*, 2015). In addition, all autophagic pathways described so far are sensitive to perturbations of intracellular or extracellular homeostasis. Thus, stimuli as different as nutritional, metabolic, chemical, physical, and hormonal cues can alter (increase or decrease) the ability of microautophagy, CMA, and macroautophagy to degrade autophagy substrates (Galluzzi *et al*, 2014; Green & Levine, 2014; Kaur & Debnath, 2015; Mukherjee *et al*, 2016; Tasset & Cuervo, 2016). The rate at which lysosomes degrade autophagy substrates is a good indicator of such a global efficiency in autophagic responses, which is commonly known as "autophagic flux" (Loos *et al*, 2014). The importance of this concept leaps to the eye upon considering macroautophagic responses and some of the biomarkers that have been employed so far to measure them, such as LC3 lipidation (as monitored by immunoblotting) and the formation of GFP-LC3$^+$ cytoplasmic dots (as monitored by immunofluorescence microscopy) (Klionsky *et al*, 2016). Both LC3 lipidation and GFP-LC3$^+$ cytoplasmic dots, indeed, are relatively reliable indicators of the pool size of the autophagosomal compartment, which is known to expand in the course of productive macroautophagic responses (increased on-rate) (Klionsky *et al*, 2016). However, autophagosomes also accumulate when the formation of autolysosomes or lysosomal degradation is blocked (decreased off-rate), a situation in which autophagy substrates are not disposed of (Boya *et al*, 2005; Gonzalez-Polo *et al*, 2005). Moreover, it cannot be excluded that the autophagosomal compartment also mediates autophagy-independent functions. Although several techniques are currently available to monitor autophagic flux in real time (Katayama *et al*, 2011; Kaizuka *et al*, 2016) and to discriminate between situations of increased on-rate and situations of decreased off-rate (Klionsky *et al*, 2016), this profound difference should be kept under critical consideration. In summary, the term *autophagic flux* refers to the rate at which the molecular machinery for autophagy identifies, segregates, and disposes of its substrates (through lysosomal degradation).

Autophagy-dependent cell death

Since the very beginning of the field, when microscopy was the main (if not the sole) experimental approach for the study of cell biology, scientists have been observing cells that die as they accumulate autophagosomes and autolysosomes in the cytoplasm (Schweichel & Merker, 1973; Eskelinen *et al*, 2011). Morphologically, these cells differ considerably from cells undergoing apoptosis or necrosis (be it regulated or accidental), which led investigators to adopt the term "autophagic cell death" or "type II cell death" based on observational/correlational (rather than interventional/causal) grounds (Schweichel & Merker, 1973; Kroemer *et al*, 2009). With the advent of modern molecular biology, it has become clear that macroautophagy has robust cytoprotective functions in the majority of pathophysiological and experimental settings (Menzies *et al*, 2015; Galluzzi *et al*, 2016). Indeed, pharmacological inhibitors of macroautophagy as well as genetic interventions targeting various components of the macroautophagy machinery generally accelerate (rather than retard) the demise of cells experiencing perturbations of homeostasis (Boya *et al*, 2005; Yousefi *et al*, 2006; Mrschtik *et al*, 2015). Thus, RCD often occurs in the context of failing macroautophagic responses that are activated as an ultimate attempt of the cell to preserve homeostasis (Galluzzi *et al*, 2015a).

Importantly, there are numerous exceptions to this tendency, suggesting that functional macroautophagic responses or components of the machinery for macroautophagy can also: (i) have little, if any, impact on RCD (so-called non-protective autophagy) (Saleh *et al*, 2016); or (ii) etiologically contribute to RCD (at least in specific developmental or pathophysiological scenarios) (Seay & Dinesh-Kumar, 2005; Masini *et al*, 2009; Sharma *et al*, 2014; Denton *et al*, 2015). For instance, disrupting any of several *Atg* genes in *D. melanogaster*, as well as blocking autophagy initiation by modulating growth signaling, results in a failure to remove larval salivary gland and midgut tissue during metamorphosis (Berry & Baehrecke, 2007; Denton *et al*, 2009, 2013; Xu *et al*, 2015). Interestingly, larval midgut degradation, which occurs independent of caspase-dependent apoptosis, does not require all components of the macroautophagy apparatus involved in starvation-induced autophagy in the *Drosophila* fat body (Xu *et al*, 2015).

Moreover, pharmacological and genetic data indicate that a specific form of autophagy-dependent cell death involving the plasma membrane Na^+/K^+-ATPase (called "autosis") occurs in cells exposed to nutrient deprivation or a BECN1-derived peptide, as well as in the brain of newborn rodents experiencing ischemia/hypoxia (Liu *et al*, 2013; Xie *et al*, 2016). In summary, *autophagy-dependent cell death* can be defined as a form of RCD that can be retarded by pharmacological or genetic inhibition of macroautophagy. In this context, it is important to note that (i) specificity issues affect most, if not all, pharmacological agents employed so far for suppressing macroautophagic responses (Maycotte *et al*, 2012; Maes *et al*, 2014; Eng *et al*, 2016; Galluzzi *et al*, 2017b); and (ii) multiple components of the macroautophagy machinery have autophagy-independent functions (Hwang *et al*, 2012; Maskey *et al*, 2013). Thus, we recommend to favor genetic approaches and to test the involvement of at least two different proteins of the macroautophagy apparatus in a specific instance of RCD before etiologically attributing it to macroautophagy. Expressions such as "ATG5-dependent cell death" or "BECN1-dependent cell death" may be even more appropriate when the involvement of one or more

specific components of the macroautophagy apparatus has been experimentally validated in the absence of links to increased autophagic flux. *Autosis* can be functionally defined as a Na^+/K^+-ATPase-mediated form of autophagy-dependent cell death.

Cytoplasm-to-vacuole targeting pathway

The cytoplasm-to-vacuole targeting (Cvt) pathway delivers hydrolases including aminopeptidase 1 (Ape1), Ape4, and alpha-mannosidase (Ams1) to the yeast vacuole (Umekawa & Klionsky, 2012). The molecular machineries for the Cvt pathway and macroautophagy share a large number of components, including several Atg proteins (Scott *et al*, 1996, 2000, 2001). Moreover, Ape1, Ape4, and Ams1 are imported into the vacuole as large oligomers, being reminiscent of the substrates of aggrephagy (Bertipaglia *et al*, 2016). The Cvt pathway, however, contributes to the preservation of normal enzymatic activity within the vacuole, especially in vegetative conditions, *de facto* mediating biosynthetic, rather than catabolic, functions (Umekawa & Klionsky, 2012). Thus, the Cvt pathway does not represent an instance of autophagy strictly speaking.

LC3-associated phagocytosis

LC3-associated phagocytosis (LAP) describes the recruitment of some (but not all) components of the macroautophagy apparatus (notably, LC3) to single-membraned phagosomes that contain extracellular pathogens or dead cell corpses destined to lysosomal degradation (Sanjuan *et al*, 2007; Martinez *et al*, 2015, 2016). Multiple molecular determinants of LAP are also required for macroautophagic responses. This applies to ATG3, ATG5, ATG7, ATG12, ATG16L1, BECN1, VPS34, and UVRAG (Martinez *et al*, 2015, 2016). However, in the mammalian systems investigated thus far, LAP does not involve ULK1 signaling, AMBRA1 and ATG14 (which are also involved in macroautophagy), but critically depends on RUBICON and NAPDH oxidase 2 (which are dispensable for macroautophagy). LAP has been involved in the control of bacterial and fungal pathogens (Sanjuan *et al*, 2007; Zhao *et al*, 2008; Gong *et al*, 2011; Lam *et al*, 2013; Choi *et al*, 2014; Martinez *et al*, 2015; Selleck *et al*, 2015), in entosis (a variant of RCD that ensues engulfment by non-phagocytic cells) (Florey *et al*, 2011), as well as in the optimal disposal of dead cells (Martinez *et al*, 2016). However, since the substrates of LAP are extracellular entities that never enter the cytoplasm, LAP cannot be considered as a *bona fide* autophagic response.

Secretory autophagy

Multiple components of the molecular apparatus for macroautophagy including (but presumably not limited to) ATG4B, ATG5, ATG7, ATG16L1, BECN1, ULK1, LC3, p62, some SNAREs and specific members of the TRIM protein family also participate in the conventional or unconventional secretion of cytoplasmic entities (including soluble proteins with extracellular functions, potentially cytotoxic protein aggregates, secretory granules, and invading pathogens) (Manjithaya *et al*, 2010; Dupont *et al*, 2011; Shravage *et al*, 2013; Lock *et al*, 2014; Gerstenmaier *et al*, 2015; Kimura *et al*, 2017), which led to the introduction of the term "secretory autophagy" (Ponpuak *et al*, 2015). Although these non-degradative functions of the macroautophagy machinery are essential for multiple intracellular and organismal processes, including viral clearance, inflammation, and hematopoiesis, they should not be considered as *bona fide* autophagic responses. Along these lines, we encourage the use of molecularly oriented expressions such as "ATG5-dependent secretion" over potentially misleading terms including "secretory autophagy".

Crinophagy

The term crinophagy refers to the degradation of secretory material following the fusion of secretory granules with lysosomes (Marzella *et al*, 1981). This process, which has been observed in secretory cells and is distinct from zymophagy, ensures the degradation and recycling of excess/obsolete secretory granules, for instance, those that persist after a hormone-induced wave of secretion is over (Weckman *et al*, 2014). Strictly speaking, crinophagy should not be considered as a form of autophagy as the content of secretory granules is not accessible from the cytoplasm (it is contained in secretory granules, similar to endosomal or phagosomal cargoes).

Components of the autophagy machinery

Autophagy substrates (autophagic cargo)

The terms *autophagy substrates* and *autophagic cargo* can be interchangeably used to describe a large and heterogeneous set of cytoplasmic entities (of endogenous or exogenous origin) that are targeted to lysosomal degradation by autophagy (Fig 1). From a conceptual standpoint, autophagy substrates should be differentiated from autophagy receptors (see below). Indeed, both autophagy substrates and receptors are subjected to lysosomal degradation, but only the latter function as part of the autophagy apparatus (Boya *et al*, 2013; Noda & Inagaki, 2015; Zaffagnini & Martens, 2016). Of note, neither hydrolytic enzymes delivered to the vacuole via Cvt (which contribute to the preservation of enzymatic homeostasis) nor extracellular entities reaching lysosomes via the endocytic pathway (which never enter the cytoplasm) can be considered as *bona fide* autophagy substrates.

Autophagy receptors and adaptors

An *autophagy receptor* is any of the proteins that bind autophagy substrates, allow for their recognition by the autophagy machinery, and become degraded within lysosomes in the course of functional autophagic responses (Stolz *et al*, 2014). Based on this definition, HSPA8 is the main receptor for endosomal microautophagy but not for CMA (during CMA, the cytoplasmic pool of HSPA8 is not degraded) (Uytterhoeven *et al*, 2015; Morozova *et al*, 2016). In addition, dozens of proteins have been involved in the recognition of macroautophagy substrates (see above) (Rogov *et al*, 2014; Farre & Subramani, 2016). Most receptors for macroautophagy share an evolutionary conserved LC3-interacting region (LIR), which allows them to bring macroautophagy substrates in the proximity of $LC3^+$ forming autophagosomes. This applies to p62, NBR1, OPTN, NDP52, BNIP3, BNIP3L, ATG34, FUNDC1, PHB2, TRIM5, TAX1BP1, Atg19, and Atg32 (Birgisdottir *et al*, 2013; Chourasia *et al*, 2015; Wei *et al*, 2017). Many macroautophagy receptors also contain ubiquitin-binding domains, allowing them to recruit ubiquitinated substrates to forming autophagosomes (Khaminets *et al*, 2016). Moreover, some receptors including yeast Atg19 and Atg34 as well as human p62, OPTN, and NDP52 have

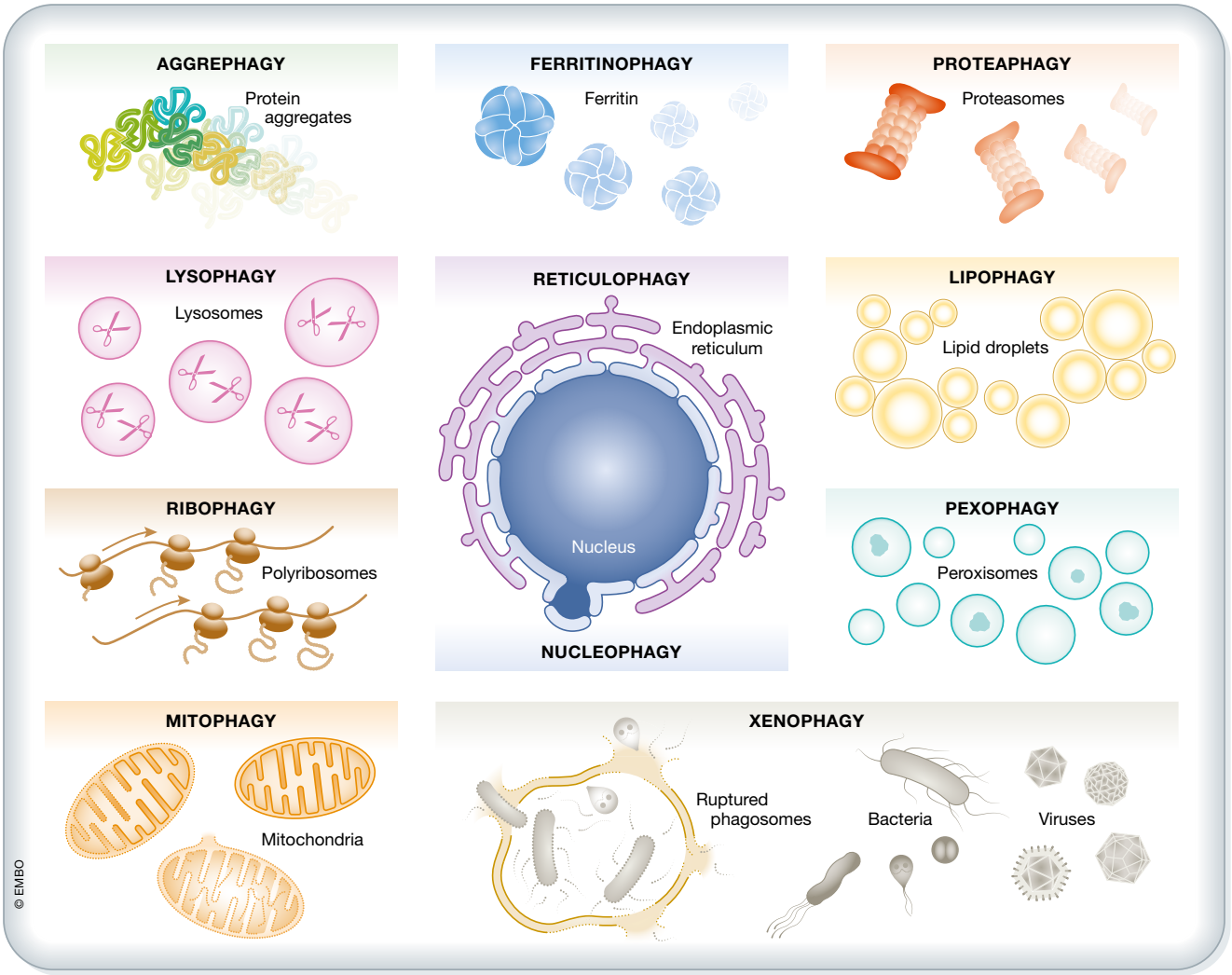

Figure 1. Autophagy substrates.
A wide and heterogeneous set of cytoplasmic entities—be they of endogenous/intracellular or exogenous/extracellular origin—can be targeted to lysosomal degradation by non-selective or selective forms of autophagy. ER, endoplasmic reticulum.

been shown to bind to the Atg12-Atg5:Atg16 (ATG12-ATG5:ATG16L1) complex to stimulate conjugation of Atg8 family members at the autophagic cargo (Fracchiolla *et al*, 2016). Along similar lines, multiple members of the TRIM protein family not only target autophagy substrates to forming autophagosomes upon LC3 binding, but also physically and functionally interact with upstream components of the autophagy apparatus, including the ULK1 and VPS34 complexes (Kimura *et al*, 2015, 2016). These proteins have been dubbed "receptor regulators". It cannot be excluded that other autophagy receptors might have regulatory functions besides cargo recognition.

Although the term *autophagy adaptor* has also been used as a synonym of autophagy receptor, we recommend to employ this expression to indicate any of the proteins that interact with Atg8 family members but are not involved in cargo recognition (and hence not degraded during macroautophagic responses) (Stolz *et al*, 2014). Two examples of autophagy adaptors outside of the ATG protein family (many members *de facto* behave as adaptors) are FYVE and coiled-coil domain containing 1 (FYCO1), which is involved in the interaction of autophagosomes with the cytoskeleton and their fusion with lysosomes, and sorting nexin 18 (SNX18), which participates in autophagosome formation (Knaevelsrud *et al*, 2013; Olsvik *et al*, 2015).

Phagophores (isolation membranes)

Phagophores (also called isolation membranes) are the precursors of autophagosomes. Mammalian phagophores generally form near ER-mitochondria contact sites in the context of unique structures staining positively for zinc finger FYVE-type containing 1 (ZFYVE1; best known as DFCP1) known as omegasomes (Axe *et al*, 2008). In mammals, phagophore biogenesis has been suggested to involve ATG9-containing vesicles that derive from the Golgi apparatus, late endosomes or the plasma membrane (Ravikumar *et al*, 2010; Orsi *et al*, 2012; Puri *et al*, 2013). Irrespective of the exact source of

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lipids (which remains a matter of debate), forming mammalian phagophores recruit the ULK1 complex and ATG14 (Karanasios *et al*, 2013), which facilitates the assembly of the autophagy-specific Class III PI3K complex (Matsunaga *et al*, 2010). This enables the association of the PI3P-binding proteins DFCP1 and WIPI2 (Polson *et al*, 2010), the formation of ATG12-ATG5:ATG16L1 complexes, and consequent local LC3 lipidation (Dooley *et al*, 2014). Either mammalian phagophores or omegasomes, or both, stain positively for ULK1, ATG13, ATG101, FIP200, VPS34, BECN1, VPS15, ATG5, ATG12, ATG16L1, DFCP1 as well as for lipidated LC3 family members (Antonioli *et al*, 2016). In yeast, phagophores are formed at the so-called "phagophore-assembly site" or "pre-autophagosomal structure" (PAS), that is, a site within the cytoplasm enriched in $Atg9^+$ vesicles with a diameter of 30–60 nm that fuse together owing to the tethering activity of Atg1 (the yeast counterpart of ULK1), Atg13, Atg17, Atg19, and Atg31 (Yamamoto *et al*, 2012; Stanley *et al*, 2014).

Autophagosomes

Transient, double-membraned organelles (mean diameter in mammals 0.5–1.5 μm) that mediate cargo sequestration and delivery to lysosomes in the course of macroautophagic responses (Shibutani & Yoshimori, 2014). Autophagosomes originate from, and hence share some biomarker proteins with, closing phagophores (see above). Since autophagosomes are devoid of hydrolytic activity, both ubiquitinated and non-ubiquitinated autophagy substrates, as well as autophagy receptors, can be detected in this compartment (Klionsky *et al*, 2016). LC3 is abundant at both the inner and outer membrane of forming autophagosomes. However, it is efficiently removed by Atg4 family members from the surface of closed autophagosomes (Lamb *et al*, 2013). In the course of functional macroautophagic responses, autophagosomes rapidly fuse with late endosomes or lysosomes (see below) and hence may be difficult to detect as a stable pool. This can be experimentally circumvented by inhibiting fusion or lysosomal acidification (Klionsky *et al*, 2016).

Amphisomes

Single- or double-membraned organelles that originate from the fusion of autophagosomes and (late) endosomes (Gordon & Seglen, 1988). Amphisomes contain common autophagosomal markers including lipidated LC3, as well as classical endosomal markers like RAB5, RAB7, and RAB11 (the latter of which is also required for autophagosome formation) (Fader *et al*, 2009; Chandra *et al*, 2015). Moreover, amphisomes have been proposed to contain small amounts of the lysosomal V-type ATPase, which would be responsible for progressive acidification of their lumen (Bader *et al*, 2015).

Autolysosomes

Single-membraned organelles that form in the course of macroautophagy upon fusion of autophagosomes or amphisomes and lysosomes (Klionsky *et al*, 2014). Autolysosomes are positive for lysosomal enzymes and classical endo/lysosomal markers, including LAMP1, LAMP2, and the V-type ATPase, but may display low levels of autophagosomal markers such as lipidated LC3, especially if autophagic flux is high (unless lysosomal hydrolases are pharmacologically or genetically inhibited) (Klionsky *et al*, 2014). Along similar lines, autophagic substrates and receptors are rapidly degraded within autolysosomes in conditions of elevated autophagic flux, implying that it may be difficult to reveal their presence in this compartment. Once the degradation of autophagy cargos is completed, autolysosomes contribute to the regeneration of the lysosomal pool via ALR (see above) (Yu *et al*, 2010). Of note, the term *autophagolysosome* indicates a specific type of autolysosome that forms in the course of some xenophagic responses (Klionsky *et al*,

Box 1: Key recommendations

- *Bona fide* autophagic responses deliver cytoplasmic material (of endogenous or exogenous origin) to lysosomes (or vacuoles) for degradation.
- Microautophagy is a LAMP2A-independent autophagic response that proceeds upon direct membrane invagination at the surface of the lysosome/vacuole.
- Endosomal microautophagy is an ESCRT-dependent, LAMP2A-independent autophagic response that relies on direct membrane invagination at the surface of late endosomes, occurring either as a bulk process or following HSPA8-mediated cargo recognition.
- Chaperone-mediated autophagy (CMA) is an HSPA8- and LAMP2A-dependent autophagic response that involves the translocation of substrates across the lysosomal membrane.
- Macroautophagy is a type of autophagic response that relies on the formation of autophagosomes and can be subtyped based upon dependence on specific factors (including—but not limited to—ATG proteins).
- Selective instances of autophagy should be defined based on the enrichment of a precise substrate, coupled to the requirement of specific molecular factors (such as autophagy receptors).
- Autophagic flux refers to the global efficacy of autophagic responses, which is generally well represented by the rate at which lysosomes degrade autophagy substrates.
- Autophagy-dependent cell death is a form of regulated cell death that can be retarded by pharmacological or genetic inhibition of components of the macroautophagy apparatus.
- Autosis is a Na^+/K^+-ATPase-mediated type of autophagy-dependent cell death.
- Cytoplasm-to-vacuole targeting (Cvt), LC3-associated phagocytosis (LAP), crinophagy, and instances of protein secretion that depend on components of the macroautophagy apparatus are not *bona fide* autophagic responses.
- Autophagy substrates are cytoplasmic entities (of endogenous/intracellular or exogenous/extracellular origin) delivered to lysosomal degradation by autophagy.
- Autophagy receptors are proteins that bind autophagy substrates, allow for their recognition by the autophagy machinery, and get degraded within lysosomes in the course of functional autophagic responses.
- Autophagy adaptors are proteins that interact with Atg8 family members, hence conferring additional functions to the autophagosome, but are not involved in cargo recognition.
- Phagophores (also called isolation membranes) are the precursors of autophagosomes.
- Autophagosomes are transient, double-membraned organelles that mediate cargo sequestration and delivery to lysosomes in the course of macroautophagic responses.
- Amphisomes are single- or double-membraned organelles that originate from the fusion of autophagosomes and (late) endosomes.
- Autolysosomes are single-membraned organelles that form in the course of macroautophagy upon fusion of autophagosomes or amphisomes with lysosomes.
- Autophagolysosomes are a specific type of autolysosome that forms in the course of xenophagic responses targeting intact or ruptured phagosomes.

2014). In this setting, autophagosomes can engulf entire phagosomes in the absence of membrane fusion, followed by the delivery of a double-membraned cargo (secluded by the inner autophagosomal membrane plus the phagosomal membrane) to lysosomes (Klionsky *et al*, 2014). We support the proper semantic and conceptual discrimination between autolysosomes and autophagolysosomes and at the same time discourage the incorrect use of these terms as interchangeable synonyms (which is rather common in the literature).

Concluding remarks

Throughout the past two decades, our understanding of autophagy in mechanistic and pathophysiological terms has progressed tremendously. In parallel, we unveiled a considerable therapeutic potential for molecules that target autophagy and autophagy-related processes such as LAP. Such a potential remains largely unexploited in the clinic, for reasons that relate to the complex nature of autophagic responses themselves, to the specificity of pharmacological agents developed so far, to the limitations of currently available models, as well as to the imprecise use of autophagy-related terms. Here, we attempted to provide semantic and conceptual recommendations that may help with this latter issue (Box 1). Our aim is not to provide a rigid vocabulary, but a working framework that can be revised and modified as the field evolves to address the current outstanding questions (Lindqvist *et al*, 2015). These recommendations are intended to facilitate the dissemination of results and ideas within and outside the field and eventually benefit scientific progress in this and other areas of biological/biomedical investigation.

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Author contributions

LG conceived and wrote the manuscript, centralized and integrated comments from co-authors, and revised the review upon editorial feedback. JMBSP designed the figure, performed bibliographic searches, and helped with table preparation. All authors corrected the article and provided valuable input to obtain a unified view. With the exception of LG and GK, authors are listed alphabetically, which does not reflect their relative contribution to the preparation of this article.

Conflict of interest

A.C.K. is an inventor on patents pertaining to Kras regulated metabolic pathways, redox control pathways in pancreatic cancer, targeting GOT1 as a therapeutic approach, and the autophagic control of iron metabolism. A.C.K. is on the SAB of Cornerstone Pharmaceuticals and is a founder of Vescor Therapeutics. The other authors declare that they have no conflict of interest.

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List of affiliations

Lorenzo Galluzzi[1,2,*]; Eric H Baehrecke[3]; Andrea Ballabio[4,5,6,7]; Patricia Boya[8]; José Manuel Bravo-San Pedro[2,9,10,11,12]; Francesco Cecconi[13,14,15]; Augustine M Choi[16]; Charleen T Chu[17]; Patrice Codogno[2,18,19,20]; Maria Isabel Colombo[21,22]; Ana Maria Cuervo[23]; Jayanta Debnath[24]; Vojo Deretic[25]; Ivan Dikic[26,27,28]; Eeva-Liisa Eskelinen[29]; Gian Maria Fimia[30,31]; Simone Fulda[32,33,34]; David A Gewirtz[35,36]; Douglas R Green[37]; Malene Hansen[38]; J Wade Harper[39]; Marja Jäättelä[40]; Terje Johansen[41]; Gabor Juhasz[42,43]; Alec C Kimmelman[44]; Claudine Kraft[45]; Nicholas T Ktistakis[46]; Sharad Kumar[47]; Beth Levine[48,49]; Carlos Lopez-Otin[50,51]; Frank Madeo[52,53]; Sascha Martens[45]; Jennifer Martinez[54]; Alicia Melendez[55,56]; Noboru Mizushima[57]; Christian Münz[58]; Leon O Murphy[59]; Josef M Penninger[60]; Mauro Piacentini[13,30]; Fulvio Reggiori[61]; David C Rubinsztein[62]; Kevin M Ryan[63]; Laura Santambrogio[64]; Luca Scorrano[65,66]; Anna Katharina Simon[67,68]; Hans-Uwe Simon[69]; Anne Simonsen[70]; Nektarios Tavernarakis[71,72]; Sharon A Tooze[73]; Tamotsu Yoshimori[74,75]; Junying Yuan[76,77]; Zhenyu Yue[78]; Qing Zhong[48] & Guido Kroemer[2,9,10,11,12,79,80]

[1]Department of Radiation Oncology, Weill Cornell Medical College, New York, NY, USA. [2]Université Paris Descartes/Paris V, Paris, France. [3]Department of Molecular, Cell and Cancer Biology, University of Massachusetts Medical School, Worcester, MA, USA. [4]Telethon Institute of Genetics and Medicine (TIGEM), Pozzuoli, Italy. [5]Medical Genetics, Department of Pediatrics, Federico II University, Naples, Italy. [6]Department of Molecular and Human Genetics, Baylor College of Medicine, Houston, TX, USA. [7]Jan and Dan Duncan Neurological Research Institute, Texas Children's Hospital, Houston, TX, USA. [8]Department of Cellular and Molecular Biology, Centro de Investigaciones Biológicas, CSIC, Madrid, Spain. [9]Université Pierre et Marie Curie/Paris VI, Paris, France. [10]Equipe 11 labellisée Ligue contre le Cancer, Centre de Recherche des Cordeliers, Paris, France. [11]INSERM, U1138, Paris, France. [12]Metabolomics and Cell Biology Platforms, Gustave Roussy Comprehensive Cancer Institute, Villejuif, France. [13]Department of Biology, University of Tor Vergata, Rome, Italy. [14]Unit of Cell Stress and Survival, Danish Cancer Society Research Center, Copenhagen, Denmark. [15]Department of Pediatric Hematology and Oncology, IRCCS Bambino Gesù Children's Hospital, Rome, Italy. [16]Division of Pulmonary and Critical Care Medicine, Joan and Sanford I. Weill Department of Medicine, Weill Cornell Medical College, New York, NY, USA. [17]Department of Pathology, University of Pittsburgh School of Medicine, Pittsburgh, PA, USA. [18]Institut Necker-Enfants Malades (INEM), Paris, France. [19]INSERM, U1151, Paris, France. [20]CNRS, UMR8253, Paris, France. [21]Laboratorio de Biología Celular y Molecular, Instituto de Histología y Embriología (IHEM)-CONICET, Mendoza, Argentina. [22]Facultad de Ciencias Médicas, Universidad Nacional de Cuyo, Mendoza, Argentina. [23]Department of Developmental and Molecular Biology, Albert Einstein College of Medicine, Bronx, NY, USA. [24]Department of Pathology, University of California San Francisco, San Francisco, CA, USA. [25]Department of Molecular Genetics and Microbiology, University of New Mexico Health Sciences Center, Albuquerque, NM, USA. [26]Institute of Biochemistry II, School of Medicine, Goethe University Frankfurt, Frankfurt, Germany. [27]Buchmann Institute for Molecular Life Sciences, Goethe University Frankfurt, Frankfurt Main, Germany. [28]Department of Immunology and Medical Genetics, University of Split School of Medicine, Split, Croatia. [29]Department of Biosciences, University of Helsinki, Helsinki, Finland. [30]National Institute for Infectious Diseases "L. Spallanzani" IRCCS, Rome, Italy. [31]Department of Biological and Environmental Sciences and Technologies (DiSTeBA), University of Salento, Lecce, Italy. [32]Institute for Experimental Cancer Research in Pediatrics, Goethe-University, Frankfurt, Germany. [33]German Cancer Consortium (DKTK), Heidelberg, Germany. [34]German Cancer Research Center (DKFZ), Heidelberg, Germany. [35]Department of Pharmacology and Toxicology and Medicine, Virginia Commonwealth University, Richmond, VA, USA. [36]Massey Cancer Center, Virginia Commonwealth University, Richmond, VA, USA. [37]Department of Immunology, St. Jude Children's Research Hospital, Memphis, TN, USA. [38]Sanford Burnham Prebys Medical Discovery Institute, La Jolla, CA, USA. [39]Department of Cell Biology, Harvard Medical School, Boston, MA, USA. [40]Cell Death and Metabolism Unit, Center for Autophagy, Recycling and Disease, Danish Cancer Society Research Center, Copenhagen, Denmark. [41]Molecular Cancer Research Group, Institute of Medical Biology, University of Tromsø – The Arctic University of Norway, Tromsø, Norway. [42]Department of Anatomy, Cell and Developmental Biology, Eotvos Lorand University, Budapest, Hungary. [43]Institute of Genetics, Biological Research Center of the Hungarian Academy of Sciences, Szeged, Hungary. [44]Department of Radiation Oncology, Perlmutter Cancer Center, NYU Langone Medical Center, New York, NY, USA. [45]Max F. Perutz Laboratories, Department of Biochemistry and Cell Biology, Vienna Biocenter, University of Vienna, Vienna, Austria. [46]Signalling Programme, Babraham Institute, Cambridge, UK. [47]Centre for Cancer Biology, University of South Australia, Adelaide, SA, Australia. [48]Center for Autophagy Research, Department of Internal Medicine, University of Texas Southwestern Medical Center, Dallas, TX, USA. [49]Howard Hughes Medical Institute (HHMI), Dallas, TX, USA. [50]Department de Bioquímica y Biología Molecular, Facultad de Medicina, Instituto Universitario de Oncología (IUOPA), Universidad de Oviedo, Oviedo, Spain. [51]Centro de Investigación en Red de Cáncer, Oviedo, Spain. [52]Institute of Molecular Biosciences, NAWI Graz, University of Graz, Graz, Austria. [53]BioTechMed Graz, Graz, Austria. [54]Immunity, Inflammation, and Disease Laboratory, National Institute of Environmental Health Sciences, Research Triangle Park, NC, USA. [55]Department of Biology, Queens College, Queens, NY, USA. [56]Graduate Center, City University of New York, New York, NY, USA. [57]Department of Biochemistry and Molecular Biology, Graduate School and Faculty of Medicine, The University of Tokyo, Tokyo, Japan. [58]Viral Immunobiology, Institute of Experimental Immunology, University of Zürich, Zurich, Switzerland. [59]Novartis Institutes for BioMedical Research, Cambridge, MA, USA. [60]Institute of Molecular Biotechnology of the Austrian Academy of Sciences (IMBA), Campus Vienna BioCentre, Vienna, Austria. [61]Department of Cell Biology, University Medical Center Groningen, University of Groningen, Groningen, The Netherlands. [62]Department of Medical Genetics, Cambridge Institute for Medical Research, University of Cambridge, Cambridge, UK. [63]Cancer Research UK Beatson Institute, Glasgow, UK. [64]Department of Pathology, Albert Einstein College of Medicine, Bronx, NY, USA. [65]Department of Biology, University of Padova, Padova, Italy. [66]Venetian Institute of Molecular Medicine, Padova, Italy. [67]Kennedy Institute of Rheumatology, University of Oxford, Oxford, UK. [68]MRC Human Immunology Unit, Weatherall Institute of Molecular Medicine, University of Oxford, Oxford, UK. [69]Institute of Pharmacology, University of Bern, Bern, Switzerland. [70]Department of Molecular Medicine, Institute of Basic Medical Sciences, University of Oslo, Oslo, Norway. [71]Institute of Molecular Biology and Biotechnology, Foundation for Research and Technology-Hellas, Heraklion, Greece. [72]Department of Basic Sciences, Faculty of Medicine, University of Crete, Heraklion, Greece. [73]Molecular Cell Biology of Autophagy Laboratory, The Francis Crick Institute, London, UK. [74]Department of Genetics, Graduate School of Medicine, Osaka University, Osaka, Japan. [75]Laboratory of Intracellular Membrane Dynamics, Graduate School of Frontier Biosciences Osaka University, Osaka, Japan. [76]Department of Cell Biology, Harvard Medical School, Boston, MA, USA. [77]Ludwig Cancer Center, Harvard Medical School, Boston, MA, USA. [78]Department of Neurology, Friedman Brain Institute, Icahn School of Medicine at Mount Sinai, New York, NY, USA. [79]Department of Women's and Children's Health, Karolinska Institute, Karolinska University Hospital, Stockholm, Sweden. [80]Pôle de Biologie, Hopitâl Européen George Pompidou, AP-HP, Paris, France

